IMMUNOLOGY

Exosomal delivery of NF-κB inhibitor delays LPS-induced preterm birth and modulates fetal immune cell profile in mouse models

Samantha Sheller-Miller¹, Enkhtuya Radnaa¹, Jae-Kwang Yoo², Eunsoo Kim², Kyungsun Choi², Youngeun Kim², Yuna Kim², Lauren Richardson¹, Chulhee Choi^{2,3}, Ramkumar Menon¹*

Accumulation of immune cells and activation of the pro-inflammatory transcription factor NF- κ B in feto-maternal uterine tissues is a key feature of preterm birth (PTB) pathophysiology. Reduction of the fetal inflammatory response and NF- κ B activation are key strategies to minimize infection-associated PTB. Therefore, we engineered extracellular vesicles (exosomes) to contain an NF- κ B inhibitor, termed super-repressor (SR) I κ Ba. Treatment with SR exosomes (1 × 10¹⁰ per intraperitoneal injection) after lipopolysaccharide (LPS) challenge on gestation day 15 (E15) prolonged gestation by over 24 hours (PTB ≤ E18.5) and reduced maternal inflammation ($n \ge 4$). Furthermore, using a transgenic model in which fetal tissues express the red fluorescent protein tdTomato while maternal tissues do not, we report that LPS-induced PTB in mice is associated with influx of fetal innate immune cells, not maternal, into feto-maternal uterine tissues. SR packaged in exosomes provides a stable and specific intervention for reducing the inflammatory response associated with PTB.

INTRODUCTION

Pregnancy is delicately balanced by various endocrine, paracrine, and immune systems (1, 2). Disruption of balanced activities in various fetal and maternal intrauterine tissues primes them for parturition-associated changes (3, 4). Intrauterine inflammation resulting from interrupted uterine homeostasis is one of the biologic effectors of parturition.

Parturition is associated with inflammation characterized by infiltration and activation of immune cells in both fetal and maternal uterine tissues [uterus (5), cervix (5), decidua (6), and fetal membranes (7)], along with an increase in pro-inflammatory cytokines and chemokines mediated by the pro-inflammatory transcription factor NF-KB (nuclear factor KB) and a decrease in anti-inflammatory cytokines and chemokines. Inflammation that mechanistically contributes to parturition is expedited by both endocrine and paracrine mediators when fetal growth and maturation is complete (8, 9). Premature disruption of immune homeostasis and overwhelming inflammation, due to either infection or other noninfectious risk factors before 37 weeks, often results in spontaneous preterm birth (PTB) (10). PTB and preterm prelabor rupture of the fetal membranes (pPROMs) account for a large subset of all PTBs (11). The majority of spontaneous PTBs and pPROMs are associated with intra-amniotic infection that can override immune tolerance, causing a host inflammatory response (12).

Although leukocytes in fetal and maternal tissues have been studied for decades, their origin (fetal versus maternal) is still unclear (5, 13–15). Recent reports have introduced the fetal origin of immune cells based on their presence in the amniotic fluid in PTB with intra-amniotic infection (16, 17). The gaps in knowledge are primarily due to a lack of specific protein markers to consistently

Copyright © 2021 The Authors, some rights reserved; exclusive licensee American Association for the Advancement of Science. No claim to original U.S. Government Works. Distributed under a Creative Commons Attribution NonCommercial License 4.0 (CC BY-NC).

differentiate between fetal and maternal cells in vivo. A better understanding of the mechanistic contributions of fetal immune cells to parturition pathways in fetal and maternal tissues will help to develop rational therapy to minimize inflammation and reduce the incidence of PTB.

The fetal inflammatory response due to infection and histologic chorioamnionitis (HCA) (infiltration of polymorphonuclear leukocytes to the fetal membranes) is a major determinant of neonatal mortality and morbidity, often associated with PTB. Multiple studies are now testing therapeutics that block inflammation by inhibiting the inflammatory transcription factor NF- κ B using cytokine-suppressive anti-inflammatory drugs (*18*, *19*), cell-penetrating peptides and small molecule inhibitors (*20*, *21*), nonspecific inhibitors of the canonical NF- κ B pathway (*22*, *23*), and flavonoids (*24*). Very few of these studies have progressed to clinical trials, and none are clinically in use, partly due to key pharmacological issues, including their half-life, mode of delivery, placental permeability, effectiveness in reducing the fetal inflammatory response, and their teratogenicity.

This study was conducted to address the limitations of drug delivery and the effectiveness in delaying PTB, as well as to reduce fetal-specific inflammation. Recent developments in drug delivery approaches have introduced extracellular vesicles, specifically exosomes that are 50 to 200 nm in size, as potential carriers of specific cargo (drugs). We have recently reported that extracellular vesicles (referred in this article as exosomes of 50 to 150 nm) are capable of trafficking between fetal and maternal units and they are capable of causing functional changes using a transgenic mouse model in which all fetal cells and tissues (and exosomes) express membrane-targeted red fluorescent protein (RFP) tdTomato (mT), while maternal tissues do not (*25*).

Using an innovative approach called exosomes for protein loading via optically reversible protein-protein interaction (EXPLOR), we have engineered exosomes that contain an inhibitor of NF- κ B, called super-repressor (SR) I κ B (26, 27). SR is a mutant form of I κ B that cannot be tagged for degradation by phosphorylation. Therefore, nuclear translocation of NF- κ B is inhibited, even in the presence of

¹Division of Maternal-Fetal Medicine and Perinatal Research, Department of Obstetrics and Gynecology, The University of Texas Medical Branch, Galveston, TX, USA. ²ILIAS Biologics, Incorporated, Daejeon, South Korea. ³Korea Advanced Institute of Science and Technology, Daejeon, South Korea. *Corresponding author. Email: ra2menon@utmb.edu

a pro-inflammatory stimulus, thus blocking the expression of specific NF- κ B-mediated genes (28). Using SR exosomes and the model developed to determine fetal-specific exosome trafficking, we tested the following objectives in this study: (i) to determine the efficacy of SR-loaded exosomes to delay infection-induced PTB in mouse models and (ii) to determine the effectiveness of SR in reducing the fetal and maternal inflammatory response (innate immune cell trafficking and inflammatory cytokine production) (29). We report that exosomal delivery of SR delayed lipopolysaccharide (LPS)-induced PTB, and this was associated with a reduction in fetal innate cell migration and the inflammatory response in various fetal and maternal tissues.

RESULTS

Using EXPLOR technology, we bioengineered exosomes to carry the NF+ κ B inhibitor SR I κ B and then tested their efficacy in a wellestablished model of LPS-induced PTB. After showing that SR can delay LPS-induced PTB, we determined fetal immune cell trafficking in response to SR treatment in a transgenic mouse model. In the following results, we show that LPS increases the presence of fetal immune cells, while SR reduces fetal immune cells in specific fetal and maternal tissues.

SR is encoded in exosomes from HEK293T cells

Exosomes were engineered to contain the NF- κ B inhibitor SR using a technology called EXPLOR (26). Here, we introduce SR, a mutant form of I κ B α that cannot be phosphorylated by I κ B kinases and suppresses the translocation of the NF- κ B complex to the nucleus, even in the presence of pro-inflammatory stimulation.

Exosomes carrying SR, as well as naïve exosomes [isolated from nontransfected human embryonic kidney (HEK) 293T cells], were characterized using transmission electron microscopy (TEM; Fig. 1A), where a characteristic cup-shaped morphology was found. In addition, exosome sizes and concentrations were determined using nanoparticle tracking analysis (NTA; Fig. 1B). SR and naïve exosomes were between 30 and 150 nm in size, supporting the TEM data. Western blots were performed to confirm packaging of SR in the exosomes (Fig. 1C), which was absent in naïve exosomes, as expected. Additional Western blots were performed to show the presence of exosome markers TSG101 and CD63, as well as the absence of the Golgi marker GM130 (Fig. 1C).

SR exosomes delay LPS-induced PTB

After establishing that exosomes carry SR, we tested the hypothesis that SR can delay LPS-induced preterm labor in mouse models. We tested this in a well-established CD-1 mouse model that consistently induces preterm labor within 12 to 14 hours (30). To test the effect of SR on delaying PTB, we injected either phosphate-buffered saline (PBS) or 100 µg of LPS intraperitoneally into pregnant mice on E15. At specific time points (every 2 hours), mice were intraperitoneally injected with either PBS (LPS), naïve exosomes (LPS + naïve), or SR (LPS + SR, 1×10^{10} exosomes per injection) and monitored for preterm labor (Fig. 2A). The dose of exosomes was chosen based on studies using SR exosomes in sepsis models (27). Mice injected with PBS delivered at term (n = 4, 112.5 hours after LPS; Fig. 2B and table S1), as expected, whereas all LPS mice delivered preterm ($n = 7, 11.5 \pm$ 0.929 hours after LPS injection, P < 0.0001 versus PBS). Naïve exosomes were used as controls for these experiments. Like LPS, all mice injected with LPS + naïve exosomes delivered preterm (n = 11, 12.9 ± 1.06 hours after LPS injection, P < 0.0001 versus PBS, P = 0.066 versus LPS + SR). Injection of SR exosomes after LPS delayed preterm labor ($n = 16, 36 \pm 10.2$ hours after LPS injection, P = 0.0005 versus PBS, P = 0.072 versus LPS) and led to term deliveries in ~20% of LPS + SR-injected mice.

Once efficacy of SR was established, we performed mechanistic studies to determine fetal immune cell trafficking using a model in which C57BL/6J females were mated with mT homozygous males because this model carries the gene construct to examine fetal-specific cell trafficking (25). With this model, pregnant mice were injected with either PBS or 2.5 μ g of LPS intraperitoneally into pregnant mice on E15. At specific time points, mice were injected with either PBS



Fig. 1. Characterization of engineered exosomes to contain the NF-*k*B inhibitor SR using EXPLOR technology. (A) Morphological characterization of naïve and SR exosomes using TEM shows exosomes with classic cup-shaped morphology. (B) Representative graph of NTA demonstrating similar size distributions in diluted samples of naïve and SR exosomes to determine size and concentration of exosome preparations. (C) HEK293T cells stably express the SR I*k*B, while naïve cells do not. Exosomes isolated from SR-producing cells express SR, mCherry, green fluorescence protein (GFP), as well as exosome markers TSG101 and CD63. Exosomes were negative for GM130.

SCIENCE ADVANCES | RESEARCH ARTICLE



Fig. 2. SR exosomes delay LPS-induced preterm labor. (**A**) Experimental model for infection-induced preterm labor in CD-1 and C57BL/6J mouse models. (**B**) Survival curve comparing PBS (blue), LPS (red), LPS + naïve (gray), and LPS + SR (green) treatment groups. LPS and LPS + naïve injections caused PTB within 24 hours, while LPS + SR delayed PTB by an average of 24 hours, compared to controls. For all groups, $n \ge 4$. (**C**) Maternal weight (left) and average pup weight (right) after LPS treatment were improved with SR treatment. For all groups, $n \ge 7$. Data were analyzed using a one-way analysis of variance (ANOVA) with a Tukey's post hoc test. (**D**) Images of pups from PBS, LPS, and LPS + SR at the time of collection on E16. (**E** to **G**) Fluorescence microscopy was used to colocalize mT⁻ expressing cells with the neutrophil marker Ly6G (green membrane fluorescence) in the fetal membranes of (E) PBS-injected, (F) LPS-injected, and (G) LPS + SR-injected mice. Scale bars, 10 μ m. (**H**) Quantitation of colocalization for Ly6G⁺ neutrophils in the fetal membranes show significant increase of neutrophils in LPS-injected mice compared to PBS. Neutrophil infiltration was significantly reduced when treated with SR exosomes. For all groups, $n \ge 5$. Data were analyzed using a one-way ANOVA with a Tukey's post hoc test. Photo credit: Samantha Sheller-Miller, Talar Kechichian, and Phyllis Gamble, University of Texas Medical Branch at Galveston.

or SR $(1 \times 10^{10}$ exosomes per injection). As naïve exosomes did not show any efficacy, we did not include this group in these studies. Twenty-four hours after LPS injection, mice were euthanized, and tissues were collected. LPS reduced maternal weight (-2.9 ± 0.619 g; Fig. 2C) compared to PBS (0.12 ± 0.469 g, P = 0.0009). LPS + SR treatment (-1.25 ± 0.098 g) substantially increased the maternal weight compared to LPS alone (P = 0.078). While not significant, the average pup weight and viability (based on fetal morphology and weight) were also improved with SR injection compared to LPS-injected mice (Fig. 2, C and D).

SR exosome treatment reduces neutrophil infiltration and HCA

HCA is defined by the presence of polymorphonuclear leukocyte infiltration of the fetal membranes (*31*), and these cells are thought to be primarily of maternal origin (*32*). HCA is an indicator of inflammation, and it can determine pregnancy outcomes as well as indicate neonatal morbidities (*33*). Using fluorescence microscopy, we stained the mT-expressing fetal membranes from PBS (Fig. 2E), LPS (Fig. 2F), and LPS + SR (Fig. 2G) for neutrophils by the expression of mouse lymphocyte antigen 6 complex locus G6D (Ly6G).

LPS-injected animals (0.282 \pm 0.043) had increased ratios of Ly6G⁺ cells to total cells in the fetal membranes (Fig. 2H), compared to either PBS (0.119 \pm 0.045, *P* = 0.008) or LPS + SR (0.100 \pm 0.032, *P* = 0.001).

Fetal macrophages are localized in maternal tissues

Although HCA was reduced by treatment with SR, the above experiment did not indicate whether neutrophils that infiltrated to the fetal membranes were primarily of fetal or maternal origin. To test our hypothesis that fetal innate immune cells, along with maternal immune cells, can traffic to both fetal and maternal tissues, we used our established transgenic mouse model that differentiates between fetal and maternal cells (25). In this model, all fetal tissues and cells express the RFP called tdTomato (mT) (fig. S1A) (34). First, we confirmed the mating efficiency, and when homozygous males with mT expressing (mT⁺) genes were mated with wild-type (WT) females, all fetal tissues expressed mT (fig. S1, B and C) (25). To determine fetal innate immune cell trafficking to the maternal compartment, we collected uterus, cervix, and decidua (basalis) on E16 and colocalized mT⁺ expressing cells with F4/80, a murine macrophage marker. As shown in Fig. 3, using confocal microscopy, mT⁺ (red fluorescence) and F4/80⁺ (green fluorescence) macrophages were colocalized in all maternal uterine tissues tested (Fig. 3, A to C).

Fetal cell trafficking is unaffected by LPS and SR treatment

Previous studies have shown that fetal cells can infiltrate maternal tissues during pregnancy (35). After establishing the model and determining fetal immune cell trafficking to maternal tissues, we then determined the differential immune response by fetal and maternal innate immune cells in LPS-induced preterm labor. To identify differences in fetal cell populations in maternal tissues (uterus, cervix, and decidua) and fetal tissues (placenta and fetal membranes), we digested samples to create a leukocyte-enriched single-cell suspen-

sion. Using flow cytometry, we first gated on viable cells and then identified mT⁺ and mT⁻ expressing fetal and maternal cells, respectively (fig. S2A). No difference in total fetal (mT⁺) cell infiltration (irrespective of type) was seen in any tissue (fig. S2, B to F) except the cervix, regardless of treatment. In the cervix, significant decrease in maternal cells was seen in LPS-injected mice (73.0 ± 4.97) compared to LPS + SR (88.5 ± 1.36, P = 0.048). In addition, total fetal cells were significantly decreased in LPS + SR-injected mice (11.50 ± 1.36, P = 0.049) compared to LPS-injected mice (27.0 ± 4.92).

Increase in fetal neutrophil infiltration in fetal tissues after LPS challenge is reduced with SR

To identify whether immune cells were fetal or maternal, after gating on viable cells, mT⁺ fetal cells were identified by red fluorescence, whereas maternal cells were negative (Fig. 4A). After identifying fetal and maternal cells, neutrophils (Ly6G⁺ cells) were identified and counted. In the placenta, compared to PBS, mice injected with LPS had significantly increased total (mT⁻ and mT⁺, P = 0.03) and mT^+ Ly6G⁺ cells (LPS, P = 0.006) (Fig. 4B). Similarly, compared to PBS, LPS + SR had significantly increased total (P = 0.050) and mT⁺ $Ly6G^+$ cells (P = 0.018). In the fetal membranes, LPS-injected mice had significantly higher total (P = 0.0005) and mT⁺ Ly6G⁺ (P < 0.0001) cells than PBS. Compared to LPS, LPS + SR had significantly lower total and mT^+ Ly6G⁺ cells (all P < 0.0001), indicating that treatment with SR may reduce HCA (Fig. 4C). This supports the data shown in Fig. 2 (E to G). Maternal neutrophils remained unchanged, regardless of treatment, indicating that the neutrophil response to LPS challenge in the placenta and fetal membranes is predominantly a fetal, not maternal, response. Other than total neutrophils in the decidua increasing in LPS (P = 0.04) and LPS + SR (P = 0.04) compared to PBS, neutrophil numbers remained unchanged in the uterus, decidua, and cervix, regardless of treatment (fig. S3, A to C).



Fig. 3. Fetal macrophages are localized in maternal tissues. (A to C) M ϕ marker F4/80 (green) was colocalized with mT (red), indicating fetal M ϕ in the maternal cervix (A), uterus (B), and decidua (C). Arrows in three-dimensional (3D) model of colocalization indicate fetal macrophages (mT⁺ F4/80⁺).

SCIENCE ADVANCES | RESEARCH ARTICLE



Fig. 4. Flow cytometry analysis of fetal and maternal neutrophils and NK cells in fetal and maternal tissues. (A) Gating strategy used to identify maternal (mT⁻) and fetal (mT⁺) neutrophils using Ly6G, NK cells using NK1.1, and DX5⁺ NK cells using NK1.1 and DX5. (**B**) Neutrophils in the placenta. mT⁻/Ly6G⁺ cells in the placenta did not change, regardless of treatment. Total Ly6G⁺ and mT⁺/Ly6G⁺ specific neutrophils in the placenta were significantly increased in LPS-injected mice compared to PBS- and LPS + SR–injected mice. (**C**) Neutrophils in the fetal membranes. Total and mT⁺/Ly6G⁺ cells were significantly increased in LPS-injected mice compared to PBS- and LPS + SR–injected mice. mT⁻/Ly6G⁺ cells did not change, regardless of treatment. (**D**) NK1.1⁺ and NK1.1⁺/DX5⁺ cells in the cervix. Total NK1.1⁺, mT⁺/NK1.1⁺, and total NK1.1⁺/DX5⁺ cells increased in LPS compared to PBS- and LPS + SR–injected mice. mT⁻/NK1.1⁺/DX5⁺ cells decreased in LPS + SR– compared to LPS-injected mice. No changes were seen in mT⁻ cells, regardless of treatment. (**E**) NK1.1⁺ and NK1.1⁺/DX5⁺ cells in the placenta. Total NK1.1⁺, NT1.1⁺/DX5, and mT⁺/NK1.1⁺/DX5⁺ cells increased in LPS compared to PBS- and LPS + SR–injected mice. No changes were seen in mT⁻ cells, regardless of treatment. (**F**) NK1.1⁺ and NK1.1⁺/DX5⁺ cells increased in LPS compared to PBS- and LPS + SR–injected mice. No changes were seen in mT⁻ cells, regardless of treatment. Blue, PBS; red, LPS; green, LPS + SR- for all groups, $n \ge 4$. Data are shown as means ± SEM. *P* values were calculated using a two-way ANOVA with a Tukey correction for multiple analyses. **P* ≤ 0.05, ***P* ≤ 0.001, *****P* ≤ 0.001.

The cytotoxic NK cell response to LPS challenge in fetal and maternal tissues is primarily fetal in origin and is reduced with SR treatment

Previous reports have shown that natural killer (NK) cells play a role in inflammation-induced preterm labor in mice through infiltration and activation at the feto-maternal interface (36). To test whether these infiltrated NK cells are fetal or maternal in origin, we determined fetal NK cells as mT⁺/NK1.1⁺ (also known as CD161b/CD161 in mouse) cells. Furthermore, we determined maturity of NK cells by the presence of DX5, also known as CD49b or $\alpha 2$ integrin, very late antigen-2, which denotes the transition of NK cells to a mature phenotype and has been associated with an increased cytotoxicity (*37*). In the uterus and decidua, no changes were seen in the percentages of NK1.1⁺ cell and NK1.1⁺/DX5⁺ NK cells (fig. S3, A and B), regardless of treatment or origin. However, in the cervix, compared to PBS, LPS significantly increased the percentage of total (*P* = 0.003), mT⁺/NK1.1⁺ (*P* = 0.011), and mT⁺/NK1.1⁺/DX5⁺ cells (*P* = 0.014; Fig. 4D). With SR treatment, compared to LPS, total NK1.1⁺ (P = 0.003), $mT^{+}/NK1.1^{+}$ (P = 0.032), total NK1.1⁺/DX5⁺ (P = 0.001), and $mT^{+}/$ NK1.1⁺/DX5⁺ (P = 0.045) cells were significantly decreased (Fig. 4D). In the placenta, there was a significant increase in total NK1.1⁺ cells in PBS mice compared to LPS-injected mice (P = 0.002) and a near significant reduction in total NK1.1⁺ cells after SR treatment (P = 0.065; Fig. 4E). Total NK1.1⁺/DX5⁺ cells and mT⁺/NK1.1⁺/DX5⁺ cells in the placenta of LPS-injected mice were increased compared to PBS (all P < 0.0001). Treatment with SR reduced total NK1.1⁺/DX5⁺ (P = 0.009) and mT⁺/NK1.1⁺/DX5⁺ cells (P = 0.008; Fig. 4E); however, no changes were seen in maternal NK1.1⁺/DX5⁺ cells. In the fetal membranes, total NK1.1⁺ (P = 0.0003) and mT⁺/NK1.1⁺ (P = 0.008) cells, as well as total NK1.1⁺/DX5⁺ (P = 0.016) and mT⁺/NK1.1⁺/ $DX5^+$ (P = 0.017) cells, were increased after LPS injection compared to PBS. Compared to LPS, total NK1.1⁺ (P < 0.0001) and mT⁺/NK1.1⁺ cells (P = 0.0004), as well as total NK1.1⁺/DX5⁺ (P = 0.008) and $mT^{+}/NK1.1^{+}/DX5^{+}$ (P = 0.018) cells, were significantly reduced after

treatment with SR (Fig. 4F). These results indicate that the predominant NK cell response to LPS challenge is of fetal, not maternal, origin and this was reduced with SR in both fetal and maternal tissues.

Inflammatory macrophage (M ϕ) infiltration is reduced in the uterus with SR treatment

The role of macrophages in term and preterm labor has been widely investigated (5, 38); however, the phenotype and origin of the invading M ϕ to the maternal-fetal interface before and during labor have not been determined (6, 38, 39). To better understand Md origin and phenotype, we defined our cell populations as mT⁻/F4/80⁺ and $mT^{+}/F4/80^{+}$ as maternal and fetal, respectively (fig. S4A). The phenotype was determined by the production of cytokines. M1 M ϕ , or classically activated $M\phi$, was identified by the production of proinflammatory cytokines tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β), while M2 M ϕ , or alternatively activated M ϕ , was identified by the production of anti-inflammatory cytokines IL-4 and IL-10 (fig. S4A). No changes in fetal or maternal macrophage number or phenotype were seen in the cervix (fig. S4C), placenta (fig. S4E), or fetal membranes (fig. S4F), regardless of treatment. However, in the uterus, total and mT⁻/F4/80⁺/IL-1 β ⁺/TNF- α ⁺ (M1) Mφ increased after LPS injection compared to PBS, which was reduced with SR injection (fig. S4B). This was also seen with total and $mT^{-}/F4/80^{+}/IL-10^{+}/IL-4^{+}$ (M2) M ϕ . No differences were seen in mT^{+} M1 and M2 M ϕ . In addition, a decrease in total F4/80⁺/IL-1 β ⁺/ TNF- α^+ (M1) M ϕ was seen in the decidua of LPS- and LPS + SRinjected mice compared to PBS-injected mice, and a decrease in mT⁻/ F4/80⁺/IL-1 β ⁺/TNF- α ⁺ in LPS + SR-injected mice compared to PBS-injected mice was seen (fig. S4D). These results correlate with previous reports, where M ϕ numbers were also reported to decrease before the initiation of labor in mice (40-42). Total F4/80⁺/IL-10⁺/ IL-4⁺ M ϕ also decreased in LPS- and LPS + SR-injected mice compared to PBS-injected mice, and mT⁻/F4/80⁺/IL-10⁺/IL-4⁺ M\phi decreased in LPS-injected mice compared to PBS-injected mice (fig. S4D).

No changes in mT^+M1 or $M2 M\phi$ numbers were seen, regardless of treatment or tissue, indicating that maternal macrophages, and not fetal macrophages, respond to LPS challenge in maternal tissues.

SR has little effect on fetal and maternal immune cells in nonuterine tissues

Previous studies have shown that fetal cells can migrate to maternal tissues outside the uterine compartment (*35*). Using our transgenic mouse model, we were also able to determine fetal cell trafficking to the liver, lung, and spleen of pregnant mice after LPS and LPS + SR treatment (fig. S5, A to C). While significant changes in fetal immune cell trafficking after LPS challenge were reduced with SR in maternal uterine tissues, the innate immune response in nonuterine tissues was predominantly of maternal origin and was unaffected by SR treatment in the maternal liver, lung, and spleen. This was likely due to the low number of total fetal cell counts in these tissues, specifically the spleen, which had nearly undetectable levels of fetal cells.

LPS-induced systemic inflammation mediated via master transcription activator NF- κ B has been reported to be an effector mechanism of PTB in mice (21, 43). Our results indicate that the innate immune response is primarily mediated by fetal-derived cells at the feto-maternal interface.

SR plasma concentration increases anti-inflammatory cytokines and decreases pro-inflammatory cytokines

Anti-inflammatory effects during pregnancy and parturition are often mediated by elevated IL-10 (44–47). Therefore, the concentration of the anti-inflammatory cytokine IL-10 was measured in maternal plasma samples on E15 at the time of LPS-induced preterm labor (Fig. 5A). Mice injected with PBS had nearly undetectable plasma levels of IL-10 (7.13 \pm 1.24 pg/ml). Mice injected with LPS had increased IL-10 compared to PBS-injected mice (380.81 \pm 77.30 pg/ml; P = 0.022), which was also seen with naïve exosome-injected mice (316.44 \pm 106.41 pg/ml; P = 0.0007). SR-injected mice had significantly



Fig. 5. SR reduces inflammation in maternal plasma. (**A**) Plasma IL-10 concentrations were significantly increased in all treatments compared to PBS mice. SR injection significantly increased plasma IL-10 concentrations compared to LPS alone. (**B**) Plasma IL-6 concentration was significantly decreased in PBS- and LPS + SR-injected compared to LPS-injected mice. (**C**) The plasma IL-8 concentration was significantly decreased in PBS compared to LPS-injected mice. (**D** and **E**) Plasma TNF- α and IL-1 β concentrations, while not significant, were reduced in all treatments compared to LPS. Blue, PBS; red, LPS; gray, LPS + naïve; green, LPS + SR. For all groups, $n \ge 4$. Data are shown as means ± SEM. *P* values were calculated using one-way ANOVA with a Tukey's post hoc test. * $P \le 0.05$, ** $P \le 0.001$, *** $P \le 0.0001$.

higher plasma IL-10 levels (627.43 ± 43.17 pg/ml) compared to LPS-injected (P < 0.0001) and PBS-injected (P = 0.026) mice. Proinflammatory effects during parturition have been associated with increased IL-6, IL-8, TNF- α , and IL-1 β (10). While no changes were seen in TNF- α and IL-1 β , regardless of treatment (Fig. 5, D and E), mice injected with LPS had significantly increased levels of IL-6 (19,116.50 ± 11,436.5 pg/ml; P = 0.045; Fig. 5B) and nearly significantly increased levels of IL-8 (>279,250 pg/ml; P = 0.052; Fig. 5C) compared to PBS-injected mice (18.64 ± 2.44 pg/ml and 148.79 ± 52.88 pg/ml, respectively). LPS + SR had nearly significantly decreased IL-6 (712.68 ± 507.40 pg/ml) compared to LPS-injected mice (P = 0.053; Fig. 5B), indicating that SR may function to increase the anti-inflammatory response in maternal plasma.

DISCUSSION

We report the following: (i) PTB in CD-1 mice models can be delayed using NF- κ B inhibitory drugs packaged into exosomes. This delay is associated with a reduction in fetal immune cell trafficking in placenta, fetal membranes, and cervix (Fig. 6, A and B). (ii) Sustained effects require repeated dosing of SR (every 2 hours); however, with our approach, we were able to delay PTB by an average of 24 hours. (iii) Prolongation of gestation by SR was associated with improved pup viability and reduction in inflammation (decreased IL-6 and increased IL-10 in maternal plasma). (iv) Fetal innate immune cell trafficking to maternal uterine tissues and LPS-induced PTB was dominated by fetal, not maternal, innate immune cells in fetal and maternal tissues. (v) The capacity of the fetal immune cells to respond and migrate to various fetal and maternal tissues indicates a functionally active fetal immune system in utero. (vi) Tropism of fetal innate immune cells to specific maternal uterine tissues after LPS challenge was tissue dependent.

PTB is a complex syndrome with multiple etiologies, wherein infection and the host's inflammatory response to infections are effectors of a major subset of PTBs (48). Although immune cell activation and migration are reported in fetal and maternal uterine tissues, specific contributions of the fetal innate immune cells in determining pregnancy outcomes remain elusive (49). PTB rates have not improved in the past few decades and suggest a gap in our understanding of the pathways, specifically fetal immune signaling associated with PTB, which is, in part, due to the inability to distinguish between fetal and maternal immune cells.

A delay in PTB with use of SR suggests that inhibition of the fetal innate immune response may affect the delivery of viable pups. We conclude that the fetal inflammatory response primes maternal uterine tissues to transition them to a parturition phenotype. This inflammatory response could be in response to a risk factor in PTB (e.g., infection), fetal tissue senescence, or signals of organ maturation capable of creating NF- κ B activation in the fetal membranes and placenta. While blocking the inflammation associated with such risk factors may delay PTB, not treating the underlying cause may also harm the fetus. Therefore, once efficacy of SR has been fully validated, future studies will include the use of antibiotics to address the underlying infection as well as reduce the inflammation in response to infection.

Defined as the persistence of fetal cells in maternal organs and circulation without any graft-versus-host reaction or rejection, fetal



Fig. 6. LPS-induced immune cell infiltration seen in PTB in a mouse model is predominantly a fetal response and is reduced at the feto-maternal interface with SR exosome treatment. LPS: Comparison between LPS and PBS. Cervix: LPS-injected mice had increased total NK1.1⁺ and DX5⁺ NK cells and increased fetal NK1.1⁺ cells. Uterus: LPS-injected mice had increased total and maternal M1 and M2 M ϕ . Decidua: LPS-injected mice had increased total neutrophils and decreased total M1 and M2 M ϕ , as well as decreased maternal M2 M ϕ . Placenta: LPS-injected mice had increased total neutrophils, NK cells. In addition, fetal neutrophils, NK cells, and DX5⁺ NK cells were also increased in LPS-injected mice. Fetal membranes (FM): LPS-injected mice had increased total and fetal neutrophils, increased total and fetal NK cells, and increased total and fetal DX5⁺ NK cells. Arrows indicate increase or decrease in number of cells. **P* = 0.07. Comparison between LPS and LPS + SR: Cervix: LPS + SR had decreased total and fetal NK cells and DX5⁺ NK cells. Uterus: LPS + SR had decreased total M1 and M2 M ϕ and decreased maternal M2 M ϕ . Decidua: No changes were seen in the decidua with SR treatment. Placenta: LPS + SR had decreased total DX5⁺ NK cells, and decreased total and fetal NK cells. Arrows indicate increase or decrease or decrease in number of cells, and decreased total ADX5⁺ NK cells. Arrows indicate increase or decrease in number of cells. **P* = 0.06.

microchimerism and characteristics of fetal immune cells in the maternal compartment have been studied extensively in normal and preterm pregnancies (50). However, their functional role is still unknown. The immune status is often determined after the incident (term or PTB in humans) and/or following animal sacrifice subsequent to specific experimental exposures. As real-time measurements of cell trafficking at the tissue level are not practical, the critical window during the change in immune status is often missed, especially in response to an infection. This is likely due to a lack of markers that are indicative of the fetal-specific immune response and the impracticality of longitudinal sampling of fetal biological samples during pregnancy. Thus, the fetal immune system has been an unrecognized critical aspect of maternal-fetal tolerance and the onset of uterine contractions.

Very few studies have attempted to evaluate the fetal versus maternal immune response under adverse pregnancy conditions. Studies by Gomez-Lopez et al. (17) used DNA fingerprinting and the Y-chromosome fluorescence in situ hybridization (FISH) to show that neutrophils in the amniotic fluid are from both the fetus and the mother and they can contribute to the inflammatory response associated with intra-amniotic infection. Gomez-Lopez et al. (17) highlighted the cause versus effect issue as fetal tissue samples were obtained after term or PTB, and as such, a critical window of immune status change is difficult to determine with post-delivery samples that may have been confounded with a multitude of labor-related factors, especially in response to an intra-amniotic infection. These findings support the HCA data presented in our article, indicating that fetal neutrophils are primarily responsible for the immune cell infiltration of the fetal membranes. Although we did not see a major shift in macrophage populations in our mouse models of PTB, Gomez-Lopez et al. (16) recently reported the predominance of fetal macrophages compared to maternal macrophages in the amniotic fluid of women with documented intra-amniotic infection and inflammation. A study by Filipovich et al. (51) used a genetic knockout of MyD88, one of the key components needed for NF-KB activation, in an intrauterine-injected Escherichia coli-induced preterm labor mouse model. E. coli-induced PTB in the mouse was dependent on maternal and not fetal MyD88 expression, suggesting maternal, and not fetal, inflammatory contributions to PTB. Our study suggests that the fetus can signal readiness for delivery by an influx of immune cells to the maternal uterine compartments; however, maternal uterine tissues must generate an inflammatory response to transition a quiescent myometrium and cervix to a laboring phenotype in response to these fetal immune signals. It is likely that maternal MyD88 knockout blocked PTB in the Filipovich model due to the lack of localized maternal uterine inflammation by NF-κB responder genes, such as inflammatory cytokines, cyclooxygenase-2 (COX-2), and matrix metalloproteinase 9 (MMP9). These gene activations are essential for myometrial contractility and cervical remodeling. A report by Kwon et al. (52) showed that placental inflammation is likely mediated through a Toll-like receptor (TLR)-MyD88-independent mechanism in response to LPS, which may negate the effect of a MyD88 knockout fetus. These data, therefore, create ambiguity with respect to MyD88mediated fetal versus maternal effects. These differences can be attributed to multiple factors, including mouse strain, stimulant used, and timing. Other studies, such as those from M. Johnson's group, showed that inflammation on the maternal side is not a requirement for labor but a consequence of labor, and J. Norman's group showed that decidual neutrophil infiltration is not required for PTB (53).

These reports further support our conclusion that the fetal inflammatory response primes a maternal quiescent system to transition to an active state, and maternal-specific immune activation is not necessary in these animal models (53).

Differences in animal models, the type of stimulant, and the definition of preterm and term birth should also be considered when interpreting these data. Regardless, the pattern emerging from all these reports suggests a significance of the fetal inflammatory response.

The currently used animal models cannot represent human term or preterm parturition. Injection of LPS or live bacteria, irrespective of route of administration, does not mimic infection/inflammationassociated PTB in humans. However, mouse models and other animal models have provided valuable information to help understand the mechanisms often seen in humans (specifically paracrine and immune functions) (25, 54). Although NF-KB is widely studied as a mediator of pro-inflammatory responses, it is a ubiquitous molecule involved in multiple alternative pathways, including apoptosis and cell proliferation (55). The mechanisms that lead to SR-mediated fetal innate immune cell trafficking are currently unclear. This study was restricted to innate immune cells. The adaptive immune response, either independently or induced in response to innate cell infiltration or activation, is still a mechanism associated with labor at term and preterm. The model we developed can be used to test the effect of SR on adaptive immune cells in future experiments. Use of SR, its mechanistic role, its efficacy, and its role in minimizing the fetal inflammatory response will lead to future studies in nonhuman primate models and human trials to reduce inflammation-associated preterm labor.

In summary, we report that the host inflammatory response in response to an infectious stimulus is driven by fetal innate immune cells. We tested a drug that decreases NF- κ B activation and reduces the fetal inflammatory response by decreasing innate immune cell infiltration. Maternal administration of engineered exosomes containing anti-inflammatory NF- κ B molecules reduced fetal inflammatory response, fetal innate immune cell migration, HCA, and delayed preterm delivery in a mouse model of infection.

METHODS

EXPLOR technology to engineer exosomes to contain NF-κB inhibitor SR

To produce exosomes carrying SR, HEK293T cells (American Type Culture Collection, Manassas, VA, USA, CRL-3216) were stably transfected with constructs containing SR (Fig. 7). For this, HEK293T cells stably expressing CIBN-EGFP-CD9 and srIkB-mcherry-CRY2 were established as described previously (25-27). For a detailed explanation of this method, please see the work of Choi et al. (27). Briefly, under blue light illumination, exosomes containing SR were produced and then isolated using tangential flow filtration and size exclusion chromatography (SEC). HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Welgene, Seoul, Korea) containing 10% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD, USA) and 1% penicillin-streptomycin (Gibco). HEK293T cells were transfected with pCMV-CIBN-EGFP and pCMV-srIkBmCherry-CRY2 with the lipofectamine transfection reagent (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. The cell population with high fluorescence intensity [green fluorescence protein (GFP) and mCherry] was measured, and then single-cell seeding on a 96-well plate was performed with a cell sorter (SH800 Cell Sorter, Sony, Minato, Tokyo, Japan). The best clone was chosen

SCIENCE ADVANCES | RESEARCH ARTICLE



Fig. 7. EXPLOR technology. (Top) Schematic of DNA constructs used to produce the SR IkB-loaded exosome. (Bottom) Schematic showing fusion proteins and their proposed activities of light-dependent protein-protein interactions to create SR-containing exosomes.

depending on the expression level of srI κ B-mcherry-CRY2. The cell culture supernatant from SR-producing cells, as well as cells grown under standard conditions (naïve), was harvested and centrifuged at 1000g for 15 min to remove cells and cell debris, and then passed through a 0.22- μ m polyethersulfone (PES) filter to remove any larger particles. The exosomes were isolated using molecular weight cutoff–based membrane filtration, and they were then purified by SEC chromatography.

Characterization of exosomes containing SR TEM of exosomes to determine size and morphology

Exosomes from stable cell lines were imaged using TEM to determine the morphology, according to previous work, but with some modifications (27). Briefly, 5 μ l of exosomes suspended in PBS was loaded onto glow-discharged carbon-coated copper grids (Electron Microscopy Sciences, Hatfield, USA). The grid was blotted with filter paper and then stained with 2% uranyl acetate. Samples were dried for 20 s and then viewed with Tecnai G2 Retrofit (FEI, Hillsboro, OR).

NTA to determine exosome size and concentration

NTA was performed using ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) and its corresponding software (ZetaView 8.02.28). Frozen exosomes in $1 \times$ PBS were thawed on ice. All samples were diluted between 1:100 and 1:10,000 in 0.2-µm filtered PBS between 1:100 and 1:10,000. For each measurement, two cycles were performed by scanning 11 cell positions with the following settings: focus: autofocus; camera sensitivity for all samples: 78.0; shutter: 70; cell temperature: 25°C. The instrument was cleaned between samples using filtered water. The results of the ZetaView were used to calculate the number of exosomes used for in vivo studies.

Western blot for the analysis of SR in the exosomes

To analyze the expression of exosome proteins, equal concentrations of the exosomes were mixed with sample buffer and boiled for 5 min. Antibodies targeting the following proteins were used: mCherry (Abcam, Cambridge, UK, ab125096), GFP (Cell Signaling Technology, Danvers, MA, CST2555), TSG101 (Abcam, ab228013), CD63 (Santa Cruz Biotechnology, sc-15363), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, sc-47724), and GM130 (Abcam, ab52649). Rabbit polyclonal anti-SRIκBα antibody was generated using recombinant SRIκBα peptide DRHDAGLDAMKDE and affinity chromatography (AbClon, Seoul, South Korea).

Animal care

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Medical Branch, Galveston. Mice were housed in a temperature- and humiditycontrolled facility with 12:12-hour light and dark cycles. Regular chow and drinking solution were provided ad libitum. For preterm labor studies, we used timed pregnant CD-1 mice (stock 022, Charles River, Houston, TX). For immune cell trafficking studies, we used a transgenic C57BL/6J mouse with a plasma membrane-targeted, two-color, fluorescent Cre-reporter allele, where tandem dimer Tomato (mT) fluorescence is expressed in the cell membrane of all cells and tissues (stock 007676, The Jackson Laboratory, Bar Harbor, ME). The RFP, mT, which is expressed in all cells and tissues, has increased brightness and photostability compared to other RFPs (34). Breeding was performed in our facility, in which WT C57BL/6J females (stock 000664, The Jackson Laboratory), 8 to 12 weeks old, were mated with males that were homozygous for mT (25). Female mice were checked daily between 8:00 a.m. and 9:00 a.m. for the presence of a vaginal plug, indicating gestation day 0.5 (E0.5). Females positive for a plug were housed separately from the males. Their weight was monitored, and a gain of at least 1.75 g by E10.5 confirmed pregnancy (56). Before tissue collection, animals were sacrificed by CO₂ inhalation according to the IACUC and the American Veterinary Medical Association guidelines.

LPS-induced preterm labor and SR exosome injections

On E15, equivalent to ~75% completed gestation in mice or ~28 weeks in humans, pregnant dams were intraperitoneally injected with one of the following: PBS or LPS {serotype 055:B5, Sigma-Aldrich, St. Louis, MO [100 µg for CD-1 (57) mice and 2.5 µg for WT mice (58)]}. Thirty minutes after injection, animals were intraperitoneally injected with either PBS, naïve exosomes, or SR exosomes (naïve and SR at 1×10^{10} exosomes in 100 µl). PBS/naïve/SR exosome injections were repeated every 2 hours for a total of five injections [Fig. 2A (CD-1) and Fig. 2B (WT)]. Animals were monitored for preterm delivery, which was defined as delivery of at least one pup on or before E18.5, using Wansview wireless cameras (Shenzhen, China). A subset of CD-1 mice was euthanized at the time of LPS-induced preterm labor (12 hours after injection), and plasma was collected for cytokine analysis. A subset of WT mice was euthanized 24 hours after LPS injection (E16, ~80% completed gestation in mice or ~30 weeks in humans) to determine fetal and maternal immune cell profile changes in fetal and maternal tissues.

Luminex assay to determine cytokine concentration in maternal plasma

Plasma collected from mice on E15 (at time of LPS delivery) was assayed for IL-10, IL-6, IL-8, TNF- α , and IL-1 β (n = 3 per group) using MILLIPLEX Mouse Cytokine Panel 1 (Millipore), following

the manufacturer's protocol. Standard curves were developed using duplicate samples of known-quantity recombinant proteins that were provided by the manufacturer. Sample concentrations were determined by relating the absorbance of the samples to the standard curve using linear regression analysis.

Immunofluorescent staining and colocalization of mT with macrophages and neutrophils in maternal and fetal tissues

For macrophage and neutrophil colocalization with mT, fresh tissue samples were washed in cold PBS and embedded in optimal cutting temperature (OCT) compound and stored at -80°C until use. OCTembedded tissues were cut into 10-µm sections and incubated at 55°C for 15 min before fixing with 4% paraformaldehyde for 15 min at room temperature. Slides were washed twice in 1× tris-buffered saline with 0.1% Tween 20 (TBST), and sections were incubated with blocking buffer (3% bovine serum albumin in TBST) for 1 hour at room temperature in a humidity chamber. The blocking buffer was removed and macrophages were labeled with Alexa Fluor 488-conjugated anti-F4/80 (Thermo Fisher Scientific, Hampton, NH, 50-167-58), while neutrophils were labeled with fluorescein isothiocyanateconjugated anti-Ly6G (BD Biosciences, La Jolla, CA, 551460) and fetal cells were labeled with anti-RFP conjugated to biotin (Abcam, ab34771) (all diluted 1:100 in the blocking buffer). After 1 hour of incubation at room temperature in a humidity chamber, sections were washed three times in TBST and incubated with phycoerythrinconjugated streptavidin (BD Biosciences, 554061) for 1 hour at room temperature. After washing, sections were incubated with 4',6-diamidino-2-phenylindole (DAPI) for nuclear staining for 2 min at room temperature and washed twice in TBST and once in water. Slides were air-dried at room temperature for 10 min and mounted using Mowiol 4-88 mounting medium. Images were captured using a Keyence BZ-X800 microscope (Keyence, Osaka, Osaka Prefecture, Japan). To create three-dimensional reconstructions, images were captured using a confocal microscope (Zeiss LSM 880) with Airyscan (Oberkochen, Germany). Brightness, contrast, and smoothing were applied to the entire image using Fiji (open source). To determine the ratio of neutrophils to total cells, a total of five images per treatment (PBS, LPS, and LPS + SR) and five regions of interest per image were used. The number of neutrophils divided by the total cell number was used to determine the neutrophil to total cell ratio.

Immune cell isolation from murine fetal and maternal tissues

WT females were euthanized 24 hours after injection (E16), and maternal [liver, lung, spleen, uterus (myometrium), cervix, and decidua (basalis)] and fetal (placenta and fetal membranes) tissues were collected, cleaned to remove excess fat, and washed in cold 1× PBS. Isolation of immune cells from tissues was performed as previously described (59), but with modifications. Tissues were cut into small pieces using fine scissors, and they were enzymatically digested with Accutase (Corning, Corning, NY) for 35 min at 37°C with gentle rocking. After incubation, the tubes were immediately placed on ice and strained through a 70-µm cell strainer. Tissues were washed twice with 10.0 ml of 1× PBS and centrifuged at 1250g for 10 min at 4°C. Cell pellets were resuspended in 2.0 ml of red blood cell lysis buffer, incubated for 10 min at room temperature, and centrifuged at 1250g for 10 min at room temperature. Cell pellets were resuspended in 1.0 ml of serum-free DMEM/Nutrient Mixture F-12 medium (DMEM/F12; Mediatech Inc.) and mixed gently. Cell suspensions were gently overlaid on $500 \,\mu$ l of neat FBS (Sigma-Aldrich) in polystyrene plastic tubes and centrifuged for 10 min at 1100g without the brake at room temperature. The supernatant was carefully aspirated, and the pellet was resuspended in 1.0 ml of DMEM/F12 supplemented with 10% FBS.

Immunophenotyping of fetal and maternal immune cells Neutrophil and NK cells

To determine neutrophil and NK cell trafficking to fetal and maternal tissues, cells in DMEM/F12 with 10% FBS were centrifuged at 1250g for 10 min at 4°C. Cell pellets were incubated with the CD16/CD32 antibody (BioLegend, San Diego, CA, 101302) for 10 min and incubated with zombie viability dye (BioLegend, San Diego, CA) and specific fluorophore-conjugated anti-mouse antibodies (table S2) for 30 min at 4°C in the dark. Cells were centrifuged at 600g for 10 min and run immediately on a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA).

M1 and M2 macrophages

To determine the macrophage phenotype and the trafficking to fetal and maternal tissues, cells were stimulated with phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (750 ng/ml) in the presence of GolgiStop (BD Bioscience) in DMEM/F12 with 10% FBS and incubated at 37° C in 5% CO₂ for 3 hours (60, 61). After incubation, cells were collected and centrifuged at 2000g for 10 min and incubated with anti-CD16/CD32 and anti-F4/80, as described above. After centrifugation, cells were fixed and permeabilized, and then stained with intracellular antibodies (table S2) for 45 min at room temperature. Cells were centrifuged and run immediately on a CytoFLEX flow cytometer (Beckman Coulter).

Gating strategy for immunophenotyping of cells

Total leukocytes were identified using the forward scatter (cell size) and side scatter (cell granularity) parameters. After gating on viable cells, we identified fetal cells by mT expression and maternal cells that were mT null. We then identified neutrophils by expression of Ly6G and NK cells by NK1.1 expression. Mature NK cells were further identified by expression of DX5, which has been shown to indicate that cells have increased cytotoxicity (*37*). Macrophages were identified using macrophage marker F4/80 and further characterized as pro-inflammatory, or M1 macrophages, with IL-1 β^+ and TNF- α^+ expression and anti-inflammatory, or M2 macrophages, with IL-10 and IL-4 expression.

Statistical analysis

Statistical analyses were performed using Prism 7 (GraphPad, San Diego, CA). All data are shown for $n \ge 3$ and displayed as means \pm SEM. Survival curves were generated using Prism 7. For multiplex data and preterm labor studies, the statistical significance between groups was determined using a one-way analysis of variance (ANOVA) with a Tukey's post hoc test. For immunophenotyping of flow cytometry data, the statistical significance was determined using a two-way ANOVA with a Tukey correction for multiple analysis. A *P* value of ≤ 0.05 was considered significant.

A post hoc power analysis was performed using G*Power (62) based on group means, SD, and effect size (f = 0.974 for Luminex analyses, 0.681 for maternal and fetal weight changes, 1.02 for PTB data, and 1.56 for flow cytometry data). This analysis revealed that the study had \geq 80% power for the ANOVA to detect differences between groups at a 0.05 significance level.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/4/eabd3865/DC1

View/request a protocol for this paper from Bio-protocol.

REFERENCES AND NOTES

- E. Golightly, H. N. Jabbour, J. E. Norman, Endocrine immune interactions in human parturition. *Mol. Cell. Endocrinol.* 335, 52–59 (2011).
- Z. Iliodromiti, N. Antonakopoulos, S. Sifakis, P. Tsikouras, A. Daniilidis, K. Dafopoulos, D. Botsis, N. Vrachnis, Endocrine, paracrine, and autocrine placental mediators in labor. *Hormones* 11, 397–409 (2012).
- P. Y. Ng, D. J. Ireland, J. A. Keelan, Drugs to block cytokine signaling for the prevention and treatment of inflammation induced preterm birth. *Front. Immunol.* 6, 166 (2015).
- J. Kaislasuo, S. Simpson, J. F. Petersen, G. Peng, P. Aldo, E. Lokkegaard, M. Paidas, L. Pal, S. Guller, G. Mor, IL-10 to TNFα ratios throughout early first trimester can discriminate healthy pregnancies from pregnancy losses. *Am. J. Reprod. Immunol.* 83, e13195 (2019).
- A. M. Mackler, G. lezza, M. R. Akin, P. McMillan, S. M. Yellon, Macrophage trafficking in the uterus and cervix precedes parturition in the mouse. *Biol. Reprod.* 61, 879–883 (1999).
- S. Hamilton, Y. Oomomian, G. Stephen, O. Shynlova, C. L. Tower, A. Garrod, S. J. Lye, R. L. Jones, Macrophages Infiltrate the human and rat decidua during term and preterm labor: Evidence that decidual inflammation precedes labor1. *Biol. Reprod.* 86, 1–9 (2012).
- I. Osman, A. Young, M. A. Ledingham, A. J. Thomson, F. Jordan, I. A. Greer, J. E. Norman, Leukocyte density and pro-inflammatory cytokine expression in human fetal membranes, decidua, cervix and myometrium before and during labour at term. *Mol. Hum. Reprod.* 9, 41–45 (2003).
- J. A. Keelan, M. Blumenstein, R. J. A. Helliwell, T. A. Sato, K. W. Marvin, M. D. Mitchell, Cytokines, prostaglandins and parturition—A review. *Placenta* 24 (suppl. A), S33–S46 (2003).
- C. R. Mendelson, Minireview: Fetal-maternal hormonal signaling in pregnancy and labor. *Mol. Endocrinol.* 23, 947–954 (2009).
- Y. Xu, R. Romero, D. Miller, P. Silva, B. Panaitescu, K. R. Theis, A. Arif, S. S. Hassan, N. Gomez-Lopez, Innate lymphoid cells at the human maternal-fetal interface in spontaneous preterm labor. *Am. J. Reprod. Immunol.* **79**, e12820 (2018).
- S. Beck, D. Wojdyla, L. Say, A. Pilar Bertran, M. Meraldi, J. Harris Requejo, C. Rubens, R. Menon, P. F. A. van Look, The worldwide incidence of preterm birth: A systematic review of maternal mortality and morbidity. *Bull. World Health Organ.* 88, 31–38 (2010).
- R. Romero, S. K. Dey, S. J. Fisher, Preterm labor: One syndrome, many causes. Science 345, 760–765 (2014).
- J. Halkias, E. Rackaityte, S. L. Hillman, D. Aran, V. F. Mendoza, L. R. Marshall, T. C. MacKenzie, T. D. Burt, CD161 contributes to prenatal immune suppression of IFNγ-producing PLZF⁺T cells. J. Clin. Invest. **129**, 3562–3577 (2019).
- M. S. F. Ng, T. L. Roth, V. F. Mendoza, A. Marson, T. D. Burt, Helios enhances the preferential differentiation of human fetal CD4⁺ naïve T cells into regulatory T cells. *Sci. Immunol.* 4, eaav5947 (2019).
- A. Schumacher, D. J. Sharkey, S. A. Robertson, A. C. Zenclussen, Immune cells at the fetomaternal interface: How the microenvironment modulates immune cells to foster fetal development. *J. Immunol.* 201, 325–334 (2018).
- N. Gomez-Lopez, R. Romero, Y. Leng, Y. Xu, R. Slutsky, D. Levenson, P. Pacora, E. Jung, B. Panaitescu, C.-D. Hsu, The origin of amniotic fluid monocytes/macrophages in women with intra-amniotic inflammation or infection. *J. Perinat. Med.* 47, 822–840 (2019).
- N. Gomez-Lopez, R. Romero, Y. Xu, Y. Leng, V. Garcia-Flores, D. Miller, S. M. Jacques, S. S. Hassan, J. Faro, A. Alsamsam, A. Alhousseini, H. Gomez-Roberts, B. Panaitescu, L. Yeo, E. Maymon, Are amniotic fluid neutrophils in women with intraamniotic infection and/or inflammation of fetal or maternal origin? *Am. J. Obstet. Gynecol.* **217**, 693.e1–693.e16 (2017).
- L. F. Stinson, D. J. Ireland, M. W. Kemp, M. S. Payne, S. J. Stock, J. P. Newnham, J. A. Keelan, Effects of cytokine-suppressive anti-inflammatory drugs on inflammatory activation in *ex vivo* human and ovine fetal membranes. *Reproduction* **147**, 313–320 (2014).
- D. J. Ireland, E. A. Nathan, S. Li, A. K. Charles, L. F. Stinson, M. W. Kemp, J. P. Newnham, J. A. Keelan, Preclinical evaluation of drugs to block inflammation-driven preterm birth. *Innate Immun.* 23, 20–33 (2017).
- S. Sundaram, C. R. Ashby Jr., R. Pekson, V. Sampat, R. Sitapara, L. Mantell, C.-H. Chen, H. Yen, K. Abhichandani, S. Munnangi, N. Khadtare, R. A. Stephani, S. E. Reznik, *N,N*-dimethylacetamide regulates the proinflammatory response associated with endotoxin and prevents preterm birth. *Am. J. Pathol.* **183**, 422–430 (2013).
- R. Pekson, V. Poltoratsky, S. Gorasiya, S. Sundaram, C. R. Ashby Jr., I. Vancurova, S. E. Reznik, N,N-dimethylacetamide significantly attenuates LPS- and TNFα-induced proinflammatory responses via inhibition of the nuclear factor kappa B pathway. *Mol. Med.* 22, 747–758 (2016).

- M. Lappas, K. Yee, M. Permezel, G. E. Rice, Sulfasalazine and BAY 11-7082 interfere with the nuclear factor-κB and IκB kinase pathway to regulate the release of proinflammatory cytokines from human adipose tissue and skeletal muscle *in vitro*. *Endocrinology* **146**, 1491–1497 (2005).
- L. Jiang, Q. Yan, R.-H. Liu, L. Zhang, Preventive and therapeutic effect of N-Acetyl-Lcysteine on infection-associated preterm labor in mice. *Asian Pac. J. Trop. Med.* 9, 197–200 (2016).
- C. Wall, R. Lim, M. Poljak, M. Lappas, Dietary flavonoids as therapeutics for preterm birth: Luteolin and kaempferol suppress inflammation in human gestational tissues *in vitro*. *Oxid. Med. Cell. Longev.* 2013, 485201 (2013).
- S. Sheller-Miller, K. Choi, C. Choi, R. Menon, Cyclic-recombinase-reporter mouse model to determine exosome communication and function during pregnancy. *Am. J. Obstet. Gynecol.* 221, 502.e1–502.e12 (2019).
- N. Yim, S.-W. Ryu, K. Choi, K. R. Lee, S. Lee, H. Choi, J. Kim, M. R. Shaker, W. Sun, J.-H. Park, D. Kim, W. D. Heo, C. Choi, Exosome engineering for efficient intracellular delivery of soluble proteins using optically reversible protein–protein interaction module. *Nat. Commun.* 7, 12277 (2016).
- H. Choi, Y. Kim, A. Mirzaaghasi, J. Heo, Y. N. Kim, J. H. Shin, S. Kim, N. H. Kim, E. S. Cho, J. I. Yook, T.-H. Yoo, E. Song, P. Kim, E.-C. Shin, K. Chung, K. Choi, C. Choi, Exosome-based delivery of super-repressor IkBα relieves sepsis-associated organ damage and mortality. *Sci. Adv.* 6, eaaz6980 (2020).
- Y. Song, Y. Kim, S. Ha, S. Sheller-Miller, J. Yoo, C. Choi, C. H. Park, The emerging role of exosomes as novel therapeutics: Biology, technologies, clinical applications, and the next. Am. J. Reprod. Immunol., e13329 (2020).
- S. C. Miller, R. Huang, S. Sakamuru, S. J. Shukla, M. S. Attene-Ramos, P. Shinn, D. van Leer, W. Leister, C. P. Austin, M. Xia, Identification of known drugs that act as inhibitors of NF-κB signaling and their mechanism of action. *Biochem. Pharmacol.* **79**, 1272–1280 (2010).
- O. Shynlova, A. Dorogin, Y. Li, S. Lye, Inhibition of infection-mediated preterm birth by administration of broad spectrum chemokine inhibitor in mice. *J. Cell. Mol. Med.* 18, 1816–1829 (2014).
- R. W. Redline, Inflammatory response in acute chorioamnionitis. Semin. Fetal Neonatal Med. 17, 20–25 (2012).
- J. H. Steel, K. O'donoghue, N. L. Kennea, M. H. F. Sullivan, A. D. Edwards, Maternal origin of inflammatory leukocytes in preterm fetal membranes, shown by fluorescence in situ hybridisation. *Placenta* 26, 672–677 (2005).
- T. Strunk, C. Campbell, D. Burgner, A. Charles, N. French, M. Sharp, K. Simmer, E. Nathan, D. Doherty, Histological chorioamnionitis and developmental outcomes in very preterm infants. J. Perinatol. 39, 321–330 (2019).
- M. D. Muzumdar, B. Tasic, K. Miyamichi, L. Li, L. Luo, A global double-fluorescent Cre reporter mouse. *Genesis* 45, 593–605 (2007).
- D. Nassar, K. Khosrotehrani, S. Aractingi, *Microchimerism in Mouse Pregnancy* (Elsevier, 2014), pp. 251–258.
- S. P. Murphy, N. N. Hanna, L. D. Fast, S. K. Shaw, G. Berg, J. F. Padbury, R. Romero, S. Sharma, Evidence for participation of uterine natural killer cells in the mechanisms responsible for spontaneous preterm labor and delivery. *Am. J. Obstet. Gynecol.* 200, 308.e1–308.e9 (2009).
- H. Arase, T. Saito, J. H. Phillips, L. L. Lanier, Cutting edge: The mouse NK cell-associated antigen recognized by DX5 moncoclonal antibody is CD49b (a₂ integrin, very late antigen-2). J. Immunol. **167**, 1141–1144 (2001).
- M. B. Brown, M. von Chamier, A. B. Allam, L. Reyes, M1/M2 macrophage polarity in normal and complicated pregnancy. *Front. Immunol.* 5, 606 (2014).
- T. Nagamatsu, D. J. Schust, The immunomodulatory roles of macrophages at the maternal–fetal interface. *Reprod. Sci.* 17, 209–218 (2010).
- I. Buhimschi, M. Ali, V. Jain, K. Chwalisz, R. E. Garfield, Differential regulation of nitric oxide in the rat uterus and cervix during pregnancy and labour. *Hum. Reprod.* 11, 1755–1766 (1996).
- S. Hamilton, Y. Oomomian, G. Stephen, O. Shynlova, C. L. Tower, A. Garrod, S. J. Lye, R. L. Jones, Macrophages infiltrate the human and rat decidua during term and preterm labor: Evidence that decidual inflammation precedes labor. *Biol. Reprod.* 86, 39 (2012).
- O. Shynlova, T. Nedd-Roderique, Y. Li, A. Dorogin, T. Nguyen, S. J. Lye, Infiltration of myeloid cells into decidua is a critical early event in the labour cascade and post-partum uterine remodelling. *J. Cell. Mol. Med.* **17**, 311–324 (2013).
- T. M. Lindström, P. R. Bennett, The role of nuclear factor kappa B in human labour. *Reproduction* 130, 569–581 (2005).
- S. J. Fortunato, R. Menon, K. F. Swan, S. J. Lombardi, Interleukin-10 inhibition of interleukin-6 in human amniochorionic membrane: Transcriptional regulation. *Am. J. Obstet. Gynecol.* **175**, 1057–1065 (1996).
- S. J. Fortunato, R. Menon, S. J. Lombardi, Interleukin-10 and transforming growth factor-β inhibit amniochorion tumor necrosis factor-α production by contrasting mechanisms of action: Therapeutic implications in prematurity. Am. J. Obstet. Gynecol. 177, 803–809 (1997).
- S. J. Fortunato, R. Menon, S. J. Lombardi, The effect of transforming growth factor and interleukin-10 on interleukin-8 release by human amniochorion may regulate histologic chorioamnionitis. *Am. J. Obstet. Gynecol.* **179**, 794–799 (1998).

- S. J. Fortunato, R. Menon, S. J. Lombardi, B. LaFleur, Interleukin-10 inhibition of gelatinases in fetal membranes: Therapeutic implications in preterm premature rupture of membranes. *Obstet. Gynecol.* 98, 284–288 (2001).
- R. Romero, O. Erez, J. Espinoza, Intrauterine infection, preterm labor, and cytokines. J. Soc. Gynecol. Investig. 12, 463–465 (2005).
- Z. H. Li, L.-L. Wang, H. Liu, K. P. Muyayalo, X.-B. Huang, G. Mor, A.-H. Liao, Galectin-9 alleviates LPS-induced preeclampsia-like impairment in rats via switching decidual macrophage polarization to M2 subtype. *Front. Immunol.* 9, 3142 (2018).
- D. W. Bianchi, G. K. Zickwolf, G. J. Weil, S. Sylvester, M. A. DeMaria, Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. *Proc. Natl. Acad. Sci.* U.S.A. 93, 705–708 (1996).
- Y. Filipovich, J. Klein, Y. Zhou, E. Hirsch, Maternal and fetal roles in bacterially induced preterm labor in the mouse. *Am. J. Obstet. Gynecol.* **214**, 386.e1–386.e9 (2016).
- J. Y. Kwon, P. Aldo, Y. You, J. Ding, K. Racicot, X. Dong, J. Murphy, G. Glukshtad, M. Silasi, J. Peng, L. Wen, V. M. Abrahams, R. Romero, G. Mor, Relevance of placental type I interferon beta regulation for pregnancy success. *Cell. Mol. Immunol.* **15**, 1010–1026 (2018).
- S. F. Rinaldi, R. D. Catalano, J. Wade, A. G. Rossi, J. E. Norman, Decidual neutrophil infiltration is not required for preterm birth in a mouse model of infection-induced preterm labor. *J. Immunol.* **192**, 2315–2325 (2014).
- R. McCarthy, C. Martin-Fairey, D. K. Sojka, E. D. Herzog, E. S. Jungheim, M. J. Stout, J. C. Fay, M. Mahendroo, J. Reese, J. L. Herington, E. J. Plosa, E. L. Shelton, S. K. England, Mouse models of preterm birth: Suggested assessment and reporting guidelines. *Biol. Reprod.* 99, 922–937 (2018).
- D. C. Guttridge, C. Albanese, J. Y. Reuther, R. G. Pestell, A. S. Baldwin Jr., NF-xB controls cell growth and differentiation through transcriptional regulation of cyclin D1. *Mol. Cell. Biol.* 19, 5785–5799 (1999).
- G. W. Heyne, E. H. Plisch, C. G. Melberg, E. P. Sandgren, J. A. Peter, R. J. Lipinski, A simple and reliable method for early pregnancy detection in Inbred Mice. J. Am. Assoc. Lab. Anim. Sci. 54, 368–371 (2015).
- B. C. Timmons, J. Reese, S. Socrate, N. Ehinger, B. C. Paria, G. L. Milne, M. L. Akins, R. J. Auchus, D. M. Intire, M. House, M. Mahendroo, Prostaglandins are essential for cervical ripening in LPS-mediated preterm birth but not term or antiprogestin-driven preterm ripening. *Endocrinology* **155**, 287–298 (2014).
- T. E. Sussan, K. Sudini, C. C. Talbot Jr., X. Wang, M. Wills-Karp, I. Burd, S. Biswal, Nrf2 regulates gene-environment interactions in an animal model of intrauterine inflammation: Implications for preterm birth and prematurity. *Sci. Rep.* 7, 40194 (2017).
- M. Arenas-Hernandez, E. N. Sanchez-Rodriguez, T. N. Mial, S. A. Robertson, N. Gomez-Lopez, Isolation of leukocytes from the murine tissues at the maternal-fetal interface. *J. Vis. Exp.*, e52866 (2015).

- W. Ai, H. Li, N. Song, L. Li, H. Chen, Optimal method to stimulate cytokine production and its use in immunotoxicity assessment. *Int. J. Environ. Res. Public Health* **10**, 3834–3842 (2013).
- N. J. O'Neil-Andersen, D. A. Lawrence, Differential modulation of surface and intracellular protein expression by T cells after stimulation in the presence of monensin or brefeldin A. *Clin. Diagn. Lab. Immunol.* 9, 243–250 (2002).
- F. Faul, E. Erdfelder, A.-G. Lang, A Buchner, G*Power: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav. Res. Methods* 39, 175–191 (2007).

Acknowledgments: We acknowledge support by all the staff from the Maternal-Fetal Medicine and Perinatal Research Laboratory (T. Kechichian, Jaboratory manager; R. Urrabaz-Garza, research associate; P. Orise, research assistant), University of Texas Medical Branch at Galveston (UTMB), TX, USA. Specifically, we thank R. Minton for expertise in image creation, especially for the summary figure. We also thank H. Spratt, professor of biostatistics, for invaluable advice on statistical analysis used in this study. Funding: This study was funded partly by NIH/NIAID (1R21AI140249-01A1) and strategic reserve funds from UTMB to R.M. Author contributions: S.S.-M. conceived and designed the experiments, conducted the study, performed all experimental aspects of the project, analyzed and interpreted data, and prepared the manuscript. E.R. assisted in experimental aspects of the project. J.-K.Y., K.C., Yuna Kim, Youngeun Kim, E.K., and C.C. produced, characterized, and provided SR and naïve exosomes and edited the manuscript. R.M. conceived the idea, provided funds and other resources, and assisted with data interpretation and preparation of the manuscript. L.R. assisted in creating the summary figure. Competing interests: R.M. and S.S.-M. are paid consultants of ILIAS Therapeutics. R.M. is a scientific advisory board member of ILIAS Biologics Inc. A U.S. patent application related to this work has been filed in 2019 and another patent application will be filed in 2020. C.C. is the founder and shareholder, and J.-K.Y., K.C., Yuna Kim, and E.K. are minor shareholders of ILIAS Biologics Inc. All other authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 17 June 2020 Accepted 4 December 2020 Published 22 January 2021 10.1126/sciadv.abd3865

Citation: S. Sheller-Miller, E. Radnaa, J.-K. Yoo, E. Kim, K. Choi, Y. Kim, Y. Kim, L. Richardson, C. Choi, R. Menon, Exosomal delivery of NF-κB inhibitor delays LPS-induced preterm birth and modulates fetal immune cell profile in mouse models. *Sci. Adv.* **7**, eabd3865 (2021).