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SHORT COMMUNICATION

Ultrasound-Guided Intrauterine Injection of Lipopolysaccharide as a Novel Model of Preterm Birth in the Mouse

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Mouse models are used to study mechanisms that link intrauterine infection and preterm birth (PTB). To mimic intrauterine infection, lipopolysaccharide (LPS) is commonly injected into the uterus via minilaparotomy, which is invasive, and can cause PTB in control animals. We hypothesized that less-invasive ultrasound-guided intrauterine LPS injection or intravaginal LPS administration could induce PTB by stimulating an inflammatory response of the uteroplacental tissues, while minimizing PTB in control animals. On day 17 of gestation mice received LPS intravaginally (10 to 240 μg; $n = 3$ to 8) or into the uterus (20 μg) under ultrasound guidance ($n = 7$) or via laparotomy ($n = 7$). Control animals received phosphate-buffered saline (PBS; $n = 5$ to 7). Intrauterine administration of LPS, both under ultrasound guidance and via laparotomy, induced delivery earlier than in PBS control groups ($P < 0.01$). Intravaginal LPS administration did not stimulate PTB. Quantitative real-time PCR and immunohistochemistry of tissues harvested 6 hours after treatment confirmed that ultrasound-guided LPS administration induced a localized inflammatory response. Ultrasound-guided intrauterine LPS injection reliably induces PTB in the mouse and mimics the local inflammatory and immune responses observed in the more-invasive laparotomy model of inflammation-induced PTB. Ultrasound-guided intrauterine LPS injection is a useful novel model of PTB for future studies and concords with the principles of reduction, replacement, and refinement. *(Am J Pathol 2015, 185: 1201–1206; http://dx.doi.org/10.1016/j.ajpath.2015.01.009)*

Preterm birth (PTB), defined as delivery before 37 weeks of gestation, remains a major public health problem, estimated to affect 11.1% of pregnancies worldwide and is the single biggest cause of neonatal mortality and morbidity.[1] Rates of PTB have remained relatively unchanged in recent decades because of a lack of effective treatments to prevent preterm labor.[2] The development of novel therapeutic treatments is hindered by a lack of understanding of the causes of PTB in many cases.

Intrauterine infection and/or inflammation is thought to be a major cause of PTB and is estimated to be present in up to 40% of preterm deliveries.[3] Animal studies have confirmed a direct causal link between infection and inflammation and PTB, where intrauterine or systemic administration of bacterial products such as lipopolysaccharide (LPS) or proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) or IL-1β, effectively induce preterm labor.[4–8]

Given the practical and ethical issues that surround the collection of tissues from pregnant women and the testing of novel interventions without prior *in vivo* proof of concept, animal models are invaluable in improving our understanding of the mechanisms that link intrauterine infection and inflammation and PTB and to investigate the potential of novel therapeutic options for PTB. The mouse is the most

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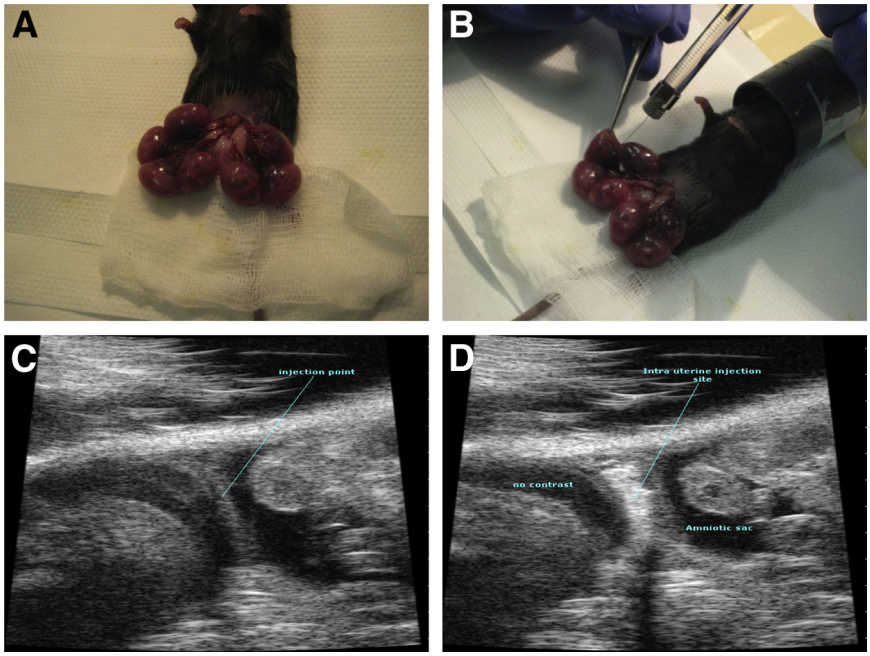

Figure 1 Models of intrauterine LPS-induced PTB. In the laparotomy model of PTB the uterine horns are exposed via minilaparotomy procedure (**A**), and PBS or LPS is injected directly into the uterus between two gestational sacs (**B**). In the ultrasound-guided model, an appropriate intrauterine space was identified sonographically (**C**), and 25 μL of ultrasound contrast agent was injected directly into the uterus, between two gestational sacs, under ultrasound guidance to verify the feasibility of the technique (**D**). LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PTB, preterm birth.

commonly used animal model in PTB research.[9,10] To recapitulate the clinical scenario in women, where the infection is usually localized to within the uteroplacental unit and subclinical, mouse models of localized intrauterine infection were developed and used successfully for many years to explore the mechanisms of PTB. To achieve this, a minilaparotomy procedure is commonly performed to expose the uterine horns, and bacterial products such as LPS are then directly injected into the uterus.[5,11–13] Although effective in achieving early parturition, this method is invasive for the animals, and laparotomy itself can result in PTB in control animals, even in the absence of LPS. (This is perhaps not surprising, given that abdominal surgery in women is associated with an increased risk of PTB.[14]) The resulting variability in outcome necessitates a relatively large sample size in experiments. In addition, we have previously reported that the laparotomy procedure induced a neutrophil influx in the connective tissue layer that surrounds the myometrial muscle bundles of mice treated with phosphate-buffered saline (PBS), which were absent in tissue from untreated control mice, highlighting that the laparotomy procedure itself can induce a rapid localized inflammatory response even without LPS administration.[11]

An alternative, less-invasive model of inflammation-induced PTB that is reported involves intravaginal LPS administration.[15–17] This model is proposed to mimic a subclinical vaginal infection, which can ascend to the cervix and induce an inflammatory response, resulting in preterm delivery.

We investigated whether less-invasive methods could reliably induce preterm labor and result in a similar local inflammatory and immune responses as was previously reported in laparotomy-based models in response to LPS,[11,13] while minimizing inflammation in the control (PBS) group. We hypothesized that being less invasive, intravaginal LPS inoculation and ultrasound-guided intrauterine LPS injection would result in fewer PTBs in the control group, while maintaining the local inflammatory response of the uteroplacental tissues of LPS-treated animals and hence effectively inducing PTB in the LPS-treated group.

Materials and Methods

Animals

All animal studies were conducted under a UK Home Office license to J.E.N. (60/4241) in accordance with the Animals Scientific Procedures Act (1986). Timed-pregnant C57Bl/6 mice were purchased from Charles River Laboratories (Margate, UK) on D9 to D11 of gestation (the day vaginal plug was found was designated as D1 of gestation). Mice were acclimatized for a minimum of 6 days before use.

Laparotomy Model of PTB

As previously described,[11,13] on D17 of gestation, a minilaparotomy procedure was performed to expose the uterine horns (Figure 1A), and mice received an intrauterine injection of LPS (20 μg; from *Escherichia coli* 0111:B4; Sigma-Aldrich, Poole, UK; $n = 7$) or sterile PBS (Gibco, Life Technologies Ltd., Paisley, UK; $n = 7$) each in a 25-μL volume by using a 33-gauge Hamilton syringe (Figure 1B).

Intravaginal Model of PTB

On D17 of gestation, mice were anesthetized with isoflurane, and mice received an intravaginal administration of LPS

(20 to 240 μg; from *E. coli* O111:B4; $n = 3$ to 4) or sterile PBS ($n = 5$) each in a volume of 40 μL by using a 200-μL pipette tip. In a separate cohort, on D16 of gestation, mice received intravaginal administration of LPS (250 μg; from *E. coli* O55:B5; Sigma-Aldrich; $n = 8$) or sterile PBS ($n = 5$) each in a volume of 100 μL by using a 200-μL pipette tip.

Ultrasound-Guided Intrauterine LPS Administration PTB Model

For all ultrasound experiments, mice were anesthetized with isoflurane (5% for induction, 1.5% for maintenance) in oxygen and were positioned supine on the ultrasound stage. Temperature and heart rate were monitored throughout all procedures. Abdominal hair was clipped, and residual hair was removed with depilatory cream, and warmed ultrasound gel was applied to the abdomen. Scans were performed with the Vevo 770 high-frequency ultrasound scanner (FUJIFILM VisualSonics, Inc., Toronto, ON, Canada) with a RMV 707B probe (center frequency, 30 MHz). The number of viable pups was determined before any procedure.

Pilot experiments were performed with only ultrasound contrast agent to confirm it was possible to use ultrasound to guide an intrauterine injection between two amniotic sacs. An appropriate space between two gestational sacs was identified sonographically (Figure 1C), and 25 μL of ultrasound contrast agent (SonoVue; sulfur hexafluoride microbubbles; Bracco UK Limited, High Wycombe, UK) was injected under direct ultrasound guidance by using a 33-gauge Hamilton syringe into the intrauterine space (Figure 1D). In a separate cohort of animals, 25 μL of methylene blue dye was injected. Animals were sacrificed and tissues were examined, which confirmed methylene blue dye was in the uterine wall and space between amniotic sacs but not in the amniotic cavity. These experiments confirmed the feasibility of using ultrasound guidance to inject directly into the uterus. We then examined the effects of intrauterine LPS administration by using the same method, replacing ultrasound contrast agent/dye with LPS or PBS. As before, on D17 of gestation, 20 μg of LPS (from *E. coli* 0111:B4; $n = 7$) or PBS ($n = 6$), each in a volume of 25 μL, was injected directly into the intrauterine space between two gestational sacs under ultrasound guidance.

Time-to-Delivery Experiments

After treatment, mice were placed in individual cages and continuously monitored with individual closed circuit television cameras and a digital video recorder. Time to delivery was recorded and defined as the number of hours from the time of LPS injection to delivery of the first pup. Within 12 to 24 hours of delivery, the number of live/dead pups was recorded, and the proportion of live born pups was calculated by dividing the number of live pups by the number of viable pups counted *in utero* either at laparotomy or via ultrasound.

Tissue Collection

In a separate cohort of mice, to determine whether ultrasound-guided intrauterine LPS administration induced a local inflammatory response in the uteroplacental tissues, mice were sacrificed by lethal exposure to carbon dioxide 6 hours after treatment with LPS ($n = 6$) or PBS ($n = 6$), and uterine, placental, and fetal membrane samples were collected and stored in RNA*later* (Sigma-Aldrich) at −80°C for quantitative real-time-PCR (qPCR) analysis. Uterine and fetal membrane samples were also fixed in 4% neutral buffered formalin and embedded in paraffin blocks for immunohistochemical analysis.

qPCR

Total RNA was extracted from the uterus, fetal membranes, and placental tissue collected 6 hours after surgery by using the RNeasy mini kit (Qiagen, Crawley, UK) as per the manufacturer's guidelines. Total RNA (300 ng) was reverse transcribed with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Life Technologies Ltd., Paisley, UK). Pre-designed gene expression assays from Applied Biosystems were used to examine the expression of *Cxcl1* (Mm04207460_m1), *Cxcl2* (Mm00436450_m1), *Ccl2* (Mm00441242_m1), *Il1b* (Mm00434228_m1), and *Tnfa* (Mm99999068_m1). Primer and probe sequences for *Actb*, *Ptgs2*, and *Il6* were designed with Primer Express software version 3.0 (Life Technologies Ltd.) as follows: *Actb* forward, 5′-GCTTCTTTGCAGCTCCTTCGT-3′; *Actb* reverse, 5′-GCGCAGCGATATCGTCATC-3′; *Actb* probe, 5′-CACCCGCCACCAGTTCGCCAT-3′; *Il6* forward, 5′-CCACGGCCTTCCCTACTTC-3′; *Il6* reverse, 5′-TGCACAACTCTTTTCTCATTCCA-3′; *Il6* probe, 5′-TCACAGAGGATACCACTCCCAACAGACCTG-3′; *Ptgs2* forward, 5′-GCTTCGGGAGCACAACAG-3′, *Ptsg2* reverse, 5′-TGGTTTGGAATAGTTGCTC-3′; and *Ptgs2* probe, 5′-TGTGCGACATACTCAAGCA-3′. Target gene expression was normalized for RNA loading by using *Actb*, and the expression in each sample was calculated relative to a calibrator sample (untreated D17 uterus, fetal membranes, or placenta), which was included in all reactions, using the $2^{-\Delta\Delta Ct}$ method of analysis. All qPCR analyses were performed on an Applied Biosystems 7900HT instrument.

Immunohistochemistry

Tissue sections (5 μm) were dewaxed in xylene and rehydrated in ethanol. Endogenous peroxidase activity was blocked by incubating slides for 30 minutes in 3% hydrogen peroxidase. To block nonspecific binding, slides were incubated for 30 minutes at room temperature in 5% normal goat serum. Sections were then incubated overnight at 4°C with rat anti−Ly-6G (dilution 1:500; BioLegend, San Diego, CA), a neutrophil surface protein. In negative control sections, the primary antibody was replaced with normal

goat serum. All sections were then incubated with the secondary antibody (ImmPRESS anti-rat IgG reagent; Vector Laboratories, Peterborough, UK) for 30 minutes at room temperature, and positive staining was detected with 3,3′-diaminobenzidine substrate for peroxidase for 5 minutes. Sections were counterstained in hematoxylin, dehydrated in ethanol, and mounted. Images were obtained with a PROVIS microscope (Olympus Optical, Hamburg, Germany) and AxioVision Rel software version 4.8 (Zeiss, Cambridge, UK).

Statistical Analysis

Data are expressed as means ± SEM. Time-to-delivery data were log-transformed before analysis, and the proportion of live born pups was arc-sin transformed before analysis. Data were analyzed by unpaired *t*-tests. All statistical analyses were performed with GraphPad Prism software version 6.0 (GraphPad, San Diego, CA). $P < 0.05$ was considered statistically significant.

Results

Intrauterine LPS Administration by Laparotomy or Ultrasound-Guided Injection Induces PTB and Reduces the Proportion of Live Born Pups

To compare and optimize an inflammatory model of PTB, three separate models were investigated. Intravaginal LPS administration was investigated because it was previously reported to reliably induce PTB.[15,16] However, we were unable to induce PTB across a wide range of doses (20 to 240 μg) by using the same LPS we have used in the intrauterine models (LPS from *E. coli* 0111:B4) (Supplemental Figure S1A). In addition, we were unable to replicate previously published studies by using a different LPS serotype (250 μg LPS from *E. coli* 055:B5) (Supplemental Figure S1B).[15,16] We therefore abandoned further attempts at the vaginal LPS model and subsequently focused our studies on two models of intrauterine inflammation-induced PTB. Intrauterine administration of 20 μg of LPS via a minilaparotomy procedure induced delivery significantly earlier than mice receiving PBS (LPS mean time to delivery of 22.75 ± 1.34 hours versus PBS mean time to delivery of 59.74 ± 9.45 hours; $P < 0.01$) (Figure 2A). Intrauterine LPS administration via minilaparotomy also resulted in a significant reduction in the proportion of live born pups compared with the PBS control group (no live pups born to LPS-treated mice; mean proportion of live born pups in PBS group, 0.46 ± 1.4; $P < 0.01$) (Figure 2B). Ultrasound-guided intrauterine LPS administration also resulted in PTB (LPS mean time to delivery of 24.75 ± 8.70 hours versus PBS mean time to delivery of 61.17 ± 3.37 hours; $P < 0.01$) (Figure 2C) and a significant reduction in the proportion of live born pups, compared with the PBS control group (LPS mean proportion of live born pups, 0.11 ± 0.11 versus 0.91 ± 0.05; $P < 0.0001$) (Figure 2D).

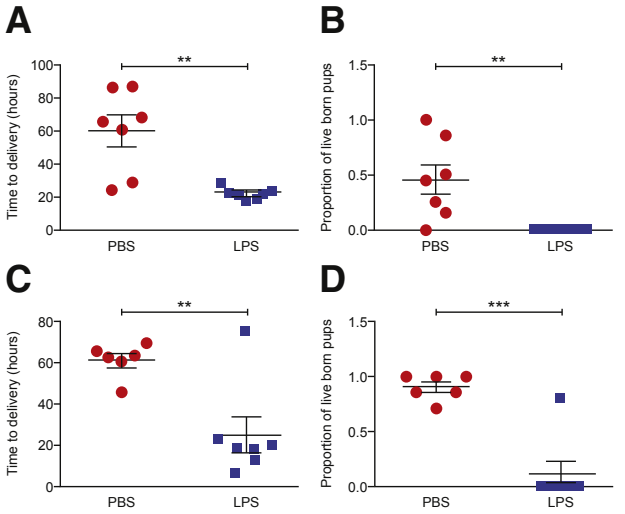

Figure 2 Effect of intrauterine LPS administration on time to delivery and the proportion of live born pups. Time to delivery (**A**) and the proportion of live born pups (**B**) were determined in mice treated with intrauterine PBS or 20 μg of LPS via minilaparotomy procedure. In the ultrasound-guided model, time to delivery (**C**) and the proportion of live born pups (**D**) were determined in mice administered intrauterine PBS or 20 μg of LPS under ultrasound guidance. Data are expressed as means ± SEM. $n = 7$ (**B**, PBS), $n = 6$ (**D**, PBS), $n = 7$ (**B** and **D**, LPS). $^{**}P < 0.01$, $^{***}P < 0.001$. LPS, lipopolysaccharide; PBS, phosphate-buffered saline.

Ultrasound-Guided Intrauterine LPS Administration Induces an Inflammatory Response in the Uteroplacental Tissues

To investigate whether ultrasound-guided intrauterine LPS administration induced a similar local inflammatory response in the uteroplacental tissues as previously described with the laparotomy model of PTB,[5,18,19] uteroplacental tissues were collected 6 hours after PBS or LPS treatment and analyzed by qPCR for the expression of several genes classically associated with the onset of parturition. In the uterus and placenta, intrauterine LPS treatment resulted in significantly elevated expression of *Cxcl1* ($P < 0.0001$), *Cxcl2* ($P < 0.0001$), *Ccl2* ($P < 0.0001$), *Il1b* ($P < 0.0001$), *Il6* ($P < 0.0001$), *Ptgs2* ($P < 0.0001$), and *Tnfa* ($P < 0.0001$) (Table 1), compared with PBS-treated mice. Similarly, in the fetal membranes intrauterine LPS administration resulted in significantly elevated expression of *Cxcl1* ($P < 0.001$), *Cxcl2* ($P < 0.0001$), *Ccl2* ($P < 0.01$), *Il1b* ($P < 0.0001$), *Ptgs2* ($P < 0.01$), and *Tnfa* ($P < 0.001$), compared with the PBS control mice. *Il6* expression in the fetal membranes was 2.39-fold higher in LPS-treated mice than in PBS-treated mice; however, this did not reach statistical significance ($P = 0.055$) (Table 1).

Neutrophil Influx in Response to Ultrasound-Guided Intrauterine LPS

To determine whether ultrasound-guided intrauterine LPS administration resulted in a neutrophil influx into the uteroplacental tissues, immunohistochemistry was performed with

Table 1 Effect of Ultrasound-Guided Intrauterine LPS Administration on mRNA Expression of Inflammatory Genes in the Uteroplacental Tissues

Tissue	Gene	Fold change versus PBS
Uterus	*Cxcl1*	414.14****
	Cxcl2	212.65****
	Ccl2	110.95****
	Il1b	47.19****
	Il6	319.17****
	Ptgs2	13.96****
	Tnfa	10.96****
Fetal membranes	*Cxcl1*	12.10***
	Cxcl2	22.90****
	Ccl2	5.20**
	Il1b	11.65****
	Il6	2.39
	Ptgs2	2.75**
	Tnfa	4.31***
Placenta	*Cxcl1*	39.13****
	Cxcl2	73.10****
	Ccl2	7.34****
	Il1b	11.85****
	Il6	19.17****
	Ptgs2	2.57****
	Tnfa	7.79****

Uterine, placental, and fetal membranes were collected 6 hours after treatment from mice administered intrauterine PBS ($n = 6$) or 20 μg of LPS ($n = 6$), under ultrasound guidance. The mRNA expression of *Cxcl1*, *Cxcl2*, *Ccl2*, *Il1b*, *Il6*, *Ptgs2*, and *Tnfa* was quantified by quantitative real-time PCR. Data are expressed as mean fold change.

$^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

an anti−Ly-6G antibody on uterine tissue and fetal membranes collected 6 hours after LPS or PBS treatment and also from untreated mice at D17 of gestation as a comparator. Neutrophils were not present in the uterine tissue of untreated mice on D17 of gestation (Figure 3A) or PBS-treated mice (Figure 3B). In uterine tissue collected from mice treated with LPS there was evidence of decidual neutrophil infiltration (Figure 3C). In the fetal membranes, no neutrophils were observed in the fetal membranes collected from untreated mice on D17 of gestation (Figure 3E) or from PBS-treated mice (Figure 3F). Although most fields were negative for Ly-6G$^+$ neutrophils in the fetal membranes of LPS-treated mice (Figure 3G), occasional Ly-6G$^+$ neutrophils were observed. Negative control sections are shown in Figure 3, D (uterus) and H (fetal membranes).

Discussion

Animal models of PTB are essential to improve our understanding of the mechanisms underlying the onset of preterm labor and for investigating the potential of treatments to delay preterm delivery and improve neonatal outcome. Here, we report the development of a novel, minimally invasive model of localized intrauterine inflammation-induced PTB, using ultrasound-guided intrauterine LPS administration.

Intrauterine LPS administration is commonly used as a model of inflammation-induced PTB.[5,11−13,19−22] However, as our data show, the traditional laparotomy method is associated with a relatively variable response, particularly in the control group. The surgical nature of this method can result in PTB in mice treated only with PBS (ie, in the control group) and subsequently affects the proportion of live born pups in this group; therefore, results are more difficult to interpret and a larger sample size is required. In comparison, we found that the use of ultrasound to guide the intrauterine injection still resulted in significantly earlier delivery in the LPS-treated mice, but importantly it did not result in any PTB in the PBS control group, and these mice had a consistently high proportion of live born pups. Minimizing the invasive nature of the surgery not only

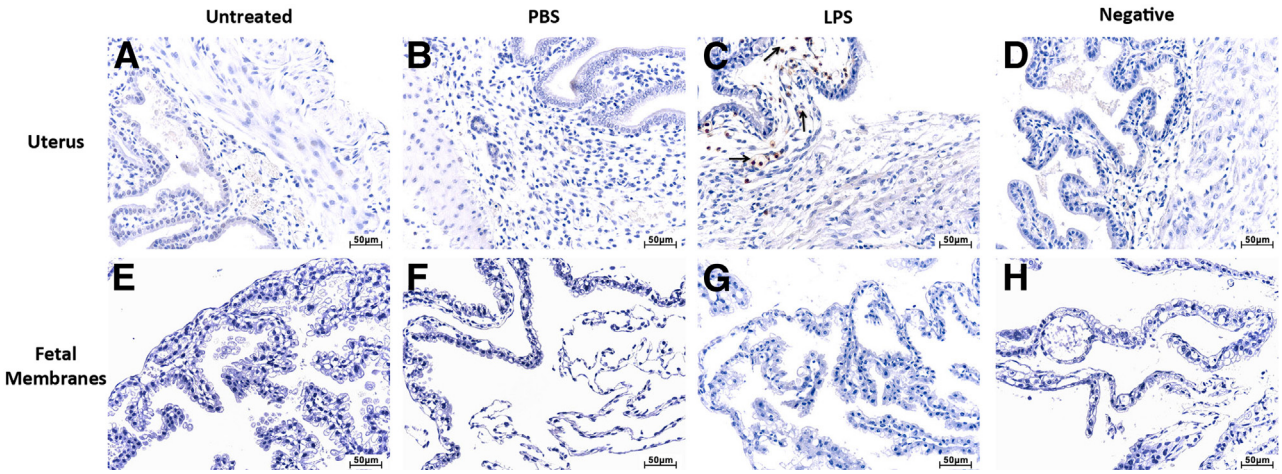

Figure 3 Effect of ultrasound-guided intrauterine LPS administration on neutrophil infiltration of the uterus and fetal membranes. Representative images of neutrophils localized by immunohistochemistry for Ly-6G in uterine tissue and fetal membranes collected 6 hours after treatment from mice administered intrauterine LPS or PBS under ultrasound guidance and from untreated mice on day 17 of gestation as a comparison. No neutrophils were observed in uterine tissue collected from untreated mice (**A**) or PBS control mice (**B**). **C:** Decidual neutrophil infiltration was observed in LPS-treated mice (**arrows**). **D:** Uterus negative control section. No neutrophils were present in fetal membranes collected from untreated (**E**) or PBS control (**F**) mice or in the majority of fields from LPS-treated mice (**G**). **H:** Fetal membrane negative control. $n = 6$ (LPS or PBS), $n = 3$ (untreated mice). Original magnification, ×20. LPS, lipopolysaccharide.

appears to reduce the variability but is also an important refinement of the model and is consistent with the principles of replacement, reduction, and refinement, which should underpin all studies that involve animals.[23]

In addition, our data show that ultrasound-guided intrauterine LPS administration results in a local inflammatory response within the uteroplacental tissues, with up-regulation of a number of inflammatory genes commonly associated with parturition and an influx of neutrophils into the decidua. These findings are in agreement with previously published studies that used the laparotomy model of intrauterine inflammation,[11–13,19,21] therefore confirming that ultrasound-guided intrauterine LPS administration results in a similar response to the invasive laparotomy model. Importantly, in contrast to our previously published studies that used the laparotomy model,[11] we did not observe neutrophil infiltration in the uterine tissue of PBS-treated mice, highlighting that the use of ultrasound-guided intrauterine injection minimizes the inflammatory response in the control animals.

Conclusion

We have shown that ultrasound-guided intrauterine LPS administration reliably induces PTB in the mouse and mimics the local inflammatory and immune response observed in other more-invasive models of inflammation-induced PTB. We believe that the development of this new minimally invasive method of inflammation-induced PTB will be useful to improve our understanding of the mechanisms underlying inflammation-induced PTB and for future analysis of novel therapeutic interventions to prevent PTB and improve neonatal outcome.

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Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2015.01.009*.

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