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The Local and Systemic Immune Response to Intrauterine LPS in the Prepartum Mouse[1]

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ABSTRACT

Inflammation plays a key role in human term and preterm labor (PTL). Intrauterine LPS has been widely used to model inflammation-induced complications of pregnancy, including PTL. It has been shown to induce an intense myometrial inflammatory cell infiltration, but the role of LPS-induced inflammatory cell activation in labor onset and fetal demise is unclear. We investigated this using a mouse model of PTL, where an intrauterine injection of 10 μg of LPS (serotype 0111:B4) was given at E16 of CD1 mouse pregnancy. This dose induced PTL at an average of 12.7 h postinjection in association with 85% fetal demise. Flow cytometry showed that LPS induced a dramatic systemic inflammatory response provoking a rapid and marked leucocyte infiltration into the maternal lung and liver in association with increased cytokine levels. Although there was acute placental inflammatory gene expression, there was no corresponding increase in fetal brain inflammatory gene expression until after fetal demise. There was marked myometrial activation of NFκB and MAPK/AP-1 systems in association with increased chemokine and cytokine levels, both of which peaked with the onset of parturition. Myometrial macrophage and neutrophil numbers were greater in the LPS-injected mice with labor onset only; prior to labor, myometrial neutrophils and monocytes numbers were greater in PBS-injected mice, but this was not associated with an earlier onset of labor. These data suggest that intrauterine LPS induces parturition directly, independent of myometrial inflammatory cell infiltration, and that fetal demise occurs without fetal inflammation. Intrauterine LPS provokes a marked local and systemic inflammatory response but with limited inflammatory cell infiltration into the myometrium or placenta.

chemokines, inflammation, leucocytes, lungs, myometrium

INTRODUCTION

Normal labor is an inflammatory process with many studies showing an increased infiltration of myeloid leukocytes to the uterus around the time of labor [1–3]. In rodent models of pregnancy, an increase in leucocyte cell numbers can also be detected in the decidua and/or myometrium during late gestation prior to labor [4–7]. Along with localized inflammation in the uterus, an increase in monocyte and neutrophil numbers has been reported in the circulation of laboring women compared to women at term, while the activation status of the systemic monocyte population has been found to be increased throughout pregnancy [8]. However, it is not clear how the inflammatory pathways involved in the initiation of labor at term are different from the infection/inflammatory induced preterm labor (PTL). Preterm birth is defined as delivery prior to 37 wk of gestation and results in the death of over 1 million babies annually [9]. PTL is the most important cause, accounting for over 66% of cases. Current treatment involves the use of tocolytics to delay delivery, allowing sufficient time for the administration of antenatal steroids to promote fetal lung maturation; however, long-term neonatal outcomes are not improved by this approach [10]. This is perhaps because intrauterine infection is a common cause of PTL, and prolonging gestation in an infected/inflammatory environment may cause greater harm to the fetus [11].

Many attempts have been made to model inflammation-induced labor, and it has been almost 20 yr since the original description of the use of intrauterine administration of *Escherichia coli* to induce PTL in the mouse [12]. This model was later adapted by Elovitz et al. [13] in 2003 using 250 μg LPS (O55:B5); although this reliably induced PTL within 48 h, it caused 100% fetal mortality. Using this model of infection-induced PTL, it has been possible to investigate leucocyte infiltration and chemokine levels in the uterus prior to and during PTL. Rinaldi et al. [14] showed that neutrophil

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chemokine expression levels and neutrophil numbers increased in the uterus following surgery, and labor induced by intrauterine LPS has been shown to have an exaggerated increase in cytokines in the myometrium compared to normal term labor and non-infection-induced PTL [6]. LPS acts via activation of toll-like receptors (TLRs), typically TLR4, which in turn activate the inflammatory cascade of transcription factors, triggering the release of chemokines, cytokines, and prolabor genes [15, 16]. The importance of the TLR4-chemokine system is shown by the failure of Gram-negative bacteria to induce PTL in TLR4 mutant mice and in wild-type mice after blocking chemokine receptors [17, 18]. We have previously shown that LPS increases COX-2 and Connexin 43 levels and induces labor in a JNK-dependent manner [16, 19]. In this study, we have used flow cytometry to investigate the changes in leucocyte kinetics locally in the uterus and placenta and systemically in the circulation, lung, and liver to understand the role that immune cells and immune modulators play in the onset of LPS-induced PTL in the mouse model. Specifically, we have tested the hypothesis that there is a relationship between labor onset, fetal demise, and changes in leucocyte populations in reproductive tissues and systemic sites in the murine model of intrauterine LPS-induced PTL.

MATERIALS AND METHODS

Animal Study Ethical Statement and Husbandry Conditions

CD1 outbred mice were purchased from Charles River at 6–8 wk of age and subsequently housed in open cages at 21 ± 1°C on a 12L:12D cycle and given Harlan Teklad 2018 Global Diet rodent food and water ad libitum. Female mice were mated overnight with males, and the day a copulatory plug was observed was designated Embryonic Day 0 (E0). Stud males were singly housed, while females were group housed (up to five dams per cage) prior to surgery, after which time they were singly housed to litter down.

The health status of all animals was monitored daily, and all mice due to enter a treatment arm were inspected prior to initiation of the protocol. Animals were monitored by infrared CCTV (RF Concepts) postsurgery and physical inspection in the lead-up to labor in case of laboring complications.

All experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986, under Home Office Licenses 70/6909 and 70/7518, with approval from the Animal Welfare and Ethical Review Boards of Imperial College and University College London. The 3Rs and ARRIVE guidelines were consulted and implemented in completion of this study and in the production of this article.

LPS-Induced Preterm Labor Model

When we established the LPS model in the group, we tested various serotypes [19] before deciding on the 0111 serotype. We also performed a dose-response curve deciding on the 10-μg dose as the being the dose that gave reliable rates of PTL with the lowest rates of maternal morbidity and mortality with some fetal survival. This dose gave similar rates of PTL as a $2–10 \times 10^3$ dose of live *E. coli* [12]. The mouse model of inflammation-induced PTL is now well established in our group [19]. Briefly, on Day 16 of gestation (E16), dams were given a subcutaneous injection of morphine (5 mg/kg) to provide suitable analgesia for a minilaparotomy performed under isoflurane anesthesia (5% isoflurane in oxygen at a 1.5-L/min flow rate). The uterine horns were exteriorized and kept moist with sterile PBS in a sterile swab. Intrauterine injections were performed on the right uterine horn between the first and second uppermost fetuses with care not to enter either amniotic cavity. Ten micrograms of *E. coli* LPS serotype 0111:B4 (Sigma) in a total volume of 25 μl or sterile PBS (25 μl) were injected. Each fetus was checked for viability before the uterine horns were replaced into the abdomen. Bupivicaine (2 mg/kg) was applied to the muscle layer and skin of the abdomen during suturing for further analgesia. Mice were allowed to recover in a heated recovery chamber, followed by single housing and regular monitoring, prior to euthanization and tissue collection. Labor time was calculated from the time of injection to the delivery of one or more fetuses. Fetal health status was assessed immediately on confirmation of the dam's death and prior to tissue collection. Blood and tissue samples, including lung, liver, myometrium, placenta, and fetal brains from the left uterine horn, were obtained at 3 and 7 h postinjection and after the birth of at least the first fetus, as initial studies suggested that delivery occurred at about 12 h post-LPS administration. Blood and tissue samples were either snap frozen for molecular analysis or processed immediately for flow cytometry.

Animals were selected for LPS or vehicle control administration randomly, with both groups injected alongside each other during every surgical session, to avoid bias. Surgeries were performed at the same time during each experimental session (between 1000 and 1200 h) to ensure that the gestational stage was as similar as possible between mice, thus reducing variation in the model. Intermediate collection time points were determined once the latency to labor post-LPS administration was calculated. Due to pregnancy rates and to again avoid collection bias, multiple time point collections were performed on the same day, while laboring samples were collected over various days. We mated enough animals to ensure that we had a minimum of six animals at each time point. The sample size was chosen based on previous in vivo work [16, 19] and the requirements for statistical normality testing. When the mating proved to be more successful than expected, we used the excess animals to increase numbers at the different time points. A single pregnant dam represents a single experimental unit throughout this study.

Tissue Collection, Cell Preparation, and Flow Cytometry

Dams were culled via exsanguination by cardiac puncture, followed by immediate cervical dislocation. Blood was immediately mixed with 5 mM EDTA and stored at 4°C until analysis within 12 h. The lungs, sections of liver, myometrium, and placentas were immediately collected and weighed. Each tissue was homogenized using a gentleMACS M tube (Miltenyi Biotec Ltd) containing 1 ml of IC fixation buffer (eBioscience) for 1 min. The homogenate was then strained through a 40-μm nylon cell strainer (BD Falcon), and the cell suspension was then centrifuged at $400 \times g$ for 5 min. Cells were then washed in PBS-FCS 2% and resuspended in permeabilizing medium PBS, 0.1% sodium azide, 0.5% BSA, and 0.2% saponin and then incubated with antibodies at 4°C for 30 min.

The fluorophore-conjugated rat antimouse MAbs antibodies used included: Ly6C (HK1.4), Gr1 (RB6-8C5), NK1.1 (PK136), F4/80 (CI:A3-1), CD45 (30-F11) (BioLegend), COX-2 (M-19) (Santa Cruz Biotechnology), CD86 (B7-2), CD11b (M1/70), MHCII (AMS-32.1), and F4/80 (BM8) (eBioscience). Levels of CD86 and COX-2 were calculated after subtraction of an isotype-matched control antibody to calculate MFI levels (IgG2a K Isotype Control [eBR2a, eBioscience catalog no. 12-4321]).

In the case of F4/80, two different antibodies were used in two separate flow panels. The cell counts were comparable between the two panels. For any data with CD86 or COX-2, MFI:F4/80 (BM8) (eBioscience catalog no. 123116) was used and for all other cell counts: F4/80 (BM8) (BioLegend catalog no. 123110) was used. The antibody was derived from the same clone but linked to different fluorophores.

After staining, lysis of red blood cells was performed using FACS lysing solution (BD). Cell counts were determined using AccuCheck counting beads (Invitrogen). Samples were run using a CyAn ADP Beckman Coulter flow cytometer following compensation with normal mouse blood using Summit software. Data were analyzed using FlowJo version 7.6.5.

Tissue Collection, Preparation, and Bio-Plex Pro Multiplex Cytokine and Cell Signaling Assays

Tissue lysates were prepared using myometrium and lung tissue snap frozen at the time of collection using a Bio-Plex Cell Lysis kit (BioRad) and a Precellys24 (Stretton Scientific Ltd) bead homogenizer according to the manufacturer's instructions. Total protein concentrations were quantified using a DC Protein Assay (BioRad). Whole blood was mixed with 5 mM EDTA immediately after cardiac puncture and centrifuged at $1000 \times g$ for 15 min. Aliquots of plasma were stored at −70°C until assayed.

Twenty-five microliters of plasma or 250 μg of total protein lysate were added per well to a Bio-Plex Pro Mouse Cytokine 23-plex Assay and completed according to the manufacturer's instructions. Analytes measured include Eotaxin, G-CSF, GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL17A, KC, CCL2, MIP-1α, MIP-1β, RANTES, and TNFα.

Levels of phosphorylated cell signaling proteins were analyzed using a Bio-Plex Pro^T cell signaling multiplex assay (BioRad). Ten micrograms of the protein lysate were incubated for 16 h with magnetic antibody-tagged beads, according to the manufacturer's instructions. Transcription factors analyzed included phosphorylated c-June (Ser63) and phosphorylated nuclear factor-κB (NFκB) p65 (Ser536). The MAPK/AP-1 pathway was assessed by measuring phosphorylated mitogen-activated protein kinase kinase 1 (MEK-1) (Ser217/Ser221), phosphorylated extracellular signal-regulated kinase (ERK)1/2 (Thr202/Tyr204, Thr185/Tyr187), phosphorylated p38 (Thr180/Tyr182), and

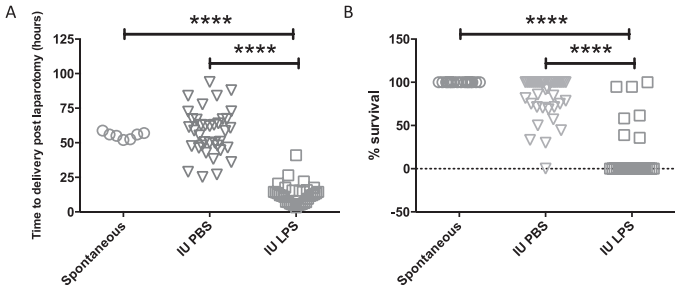

FIG. 1. **A**) Delivery time after intrauterine injection of PBS or LPS (10 μg of *E. coli* LPS serotype 0111:B4). Delivery time was recorded when at least one fetus had been delivered. For naive mice in spontaneous labor, time to delivery was recorded from 0900 h on E16. For the mice given an intrauterine injection, delivery time was counted in hours from the time of the laparotomy. The data are expressed as median interquartile range. Data were not normally distributed and were analyzed using a Kruskal-Wallis test with a Dunn multiple comparison posttest (****$P < 0.0001$; n = 16–37). **B**) Fetal survival after intrauterine injection of PBS or LPS (10 μg of *E. coli* LPS serotype 0111:B4). Fetal survival is defined as the number of fetuses born alive at delivery. The data are expressed as median interquartile range. Data were not normally distributed and were analyzed using a Kruskal-Wallis test with a Dunn multiple comparison posttest (****$P < 0.0001$; n = 16–37).

phosphorylated JNK (Thr^{183}/Tyr^{185}). The data are expressed as a ratio of mean fluorescence intensity of a sample to mean fluorescence intensity of the positive control. The positive control was supplied by the manufacturer for each of the analytes. Cytokine and cell-signaling plates were analyzed using Bio-Plex Magpix multiplex reader and Bio-Plex Manager version 6.1 software (BioRad).

BrdU Injection

To label dividing cells in vivo, 100 μl of 10-mg/ml BrdU (BD Pharmingen) in sterile PBS was administered by i.p. injection. Previous kinetic studies demonstrated Brdu labeling of bone-marrow-derived Ly6Chigh monocytes is maximal after 18 h [20] and that at 24 h after BrdU injection in E7.5 mice, >50% of the Ly-$6C^{high}$ monocytes in the circulation and in the myometrium were BrdU positive [21]. Therefore, to assess monocyte infiltration into the myometrium, we performed the laparotomy and intrauterine PBS injection at 17 h post-BrdU injection and tissues collected 7 h later at 24 h post-BrdU.

Quantitative RT-PCR Analysis

Total RNA was extracted from tissue using an RNeasy kit and Qiashredder (Qiagen Ltd). The concentration of RNA in each sample was measured by a NanoDrop ND1000 spectrophotometer (ThermoFisher Scientific). One microgram of RNA was converted to cDNA using the MuLV reverse transcriptase system (Applied Biosystems Ltd.), according to manufacturer's protocols. Primer sets were designed using Primer3 software and obtained from Life Technologies Ltd. (Table 1). Quantitative PCR was performed in the presence

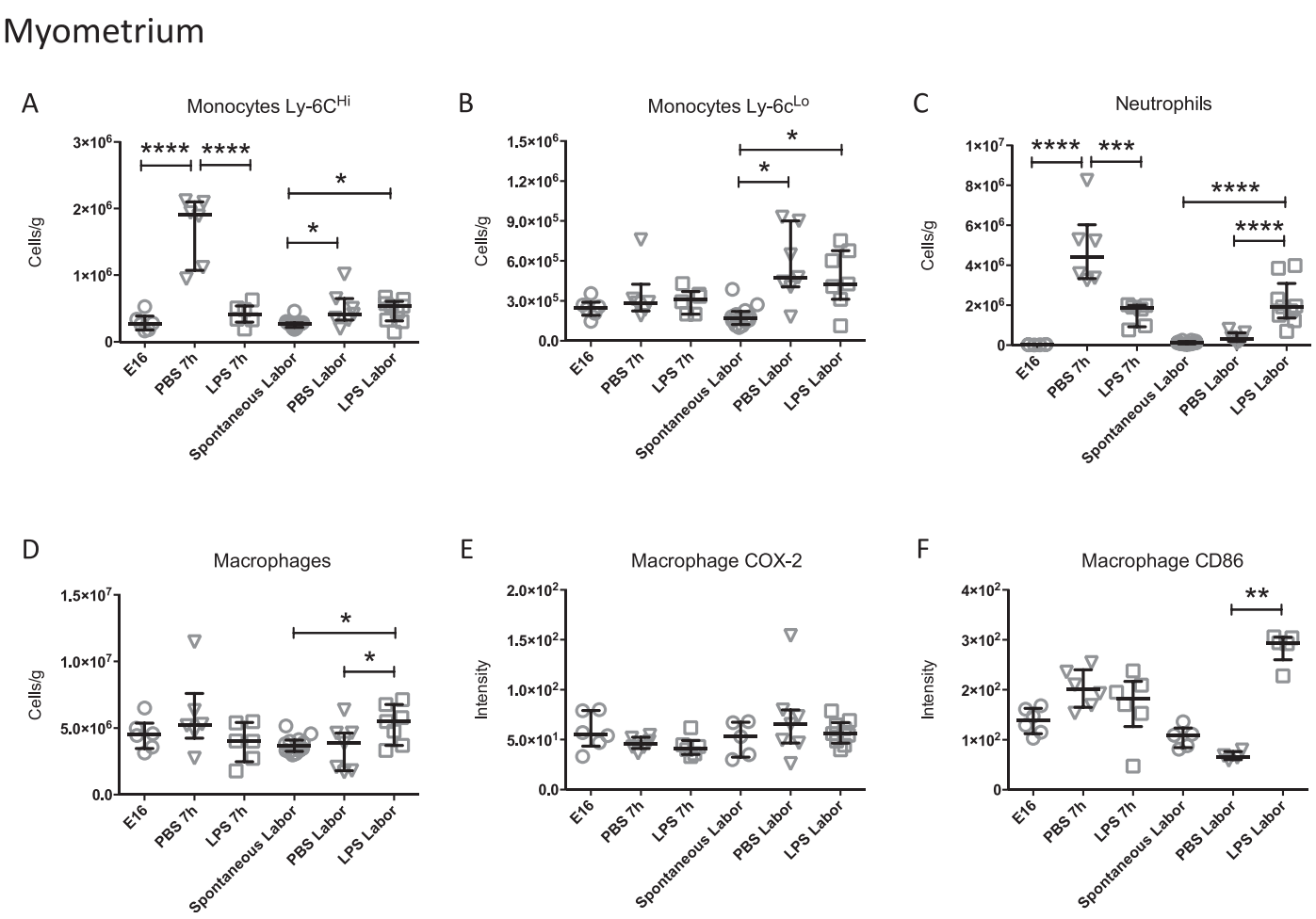

FIG. 2. **A**–**F**) Cell density of leukocytes in the myometrium and myometrial macrophage activation status in untreated mice on E16 and following a laparotomy and intrauterine injection of either PBS or LPS (10 μg of *E. coli* LPS serotype 0111:B4) at 7 h postprocedure and in labor. Cell densities of $Ly6c^{hi}$ and $Ly6c^{lo}$ monocytes (CD11b+, F/80+), neutrophils (CD11b+, Ly6G+), and myometrial macrophages (CD11b+, F4/80++, $Ly6C^{lo}$) and activation status of myometrial macrophages at each time point are expressed as median interquartile range. When the data were normally distributed, they were analyzed using a one-way ANOVA with Bonferroni posttest, and when the data were not normally distributed, they were analyzed using a Kruskal-Wallis test with a Dunn multiple comparison posttest (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$; n = 6–12).

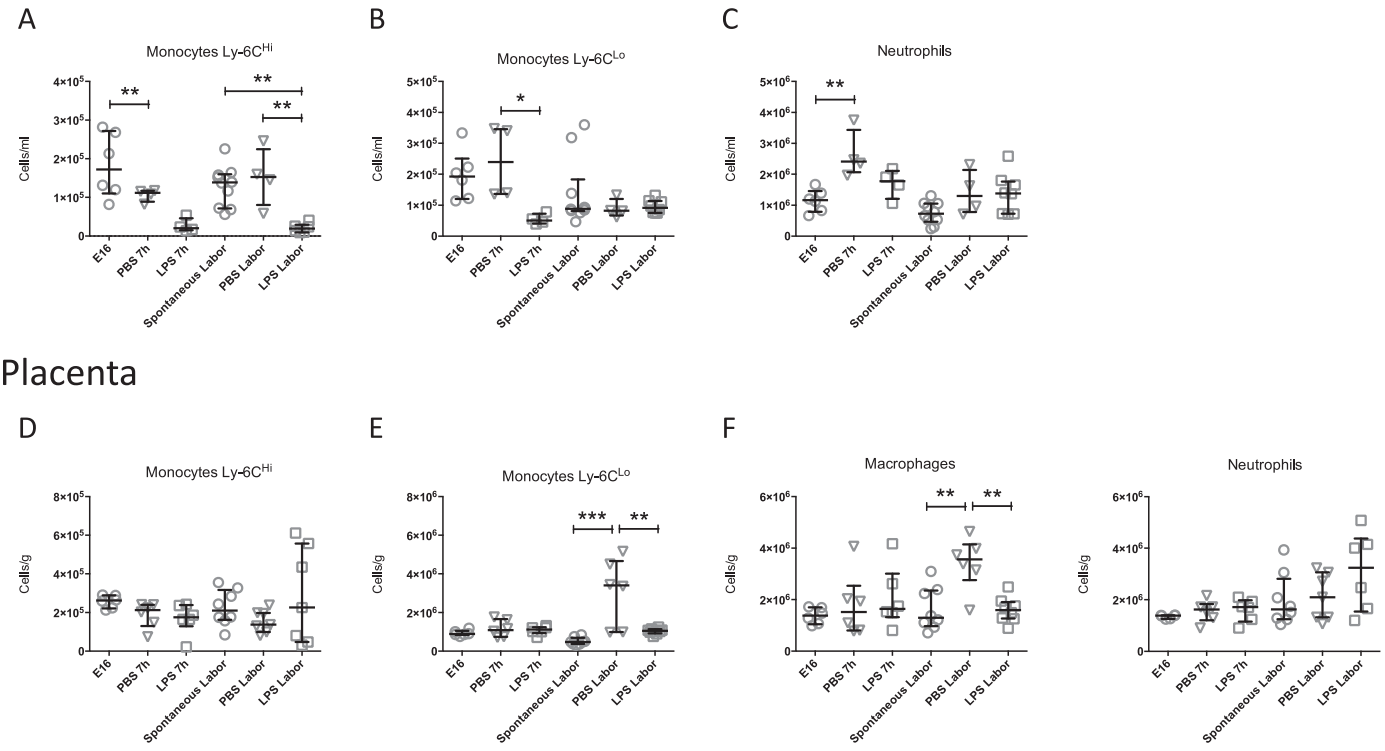

FIG. 3. Cell counts and density of leukocytes in the circulation (**A**–**C**, n = 4–10) and placenta (**D**–**G**, n = 6–8) of untreated mice on E16 and following a laparotomy and intrauterine injection of either PBS or LPS (10 μg of *E. coli* LPS serotype 0111:B4) at 7 h postprocedure and in labor. Cell densities at each time point are expressed as median interquartile range. When the data were normally distributed, they were analyzed using a one-way ANOVA with Bonferroni posttest, and when the data were not normally distributed, they were analyzed using a Kruskal-Wallis test with a Dunn multiple comparison posttest (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, n = 6–12).

of SYBR Green (Roche Diagnostics Ltd.), and amplicon yield was monitored by Rotor Gene R-G 3000 (Corbett Research).

Statistical Analysis

All data were tested for normality using a Kolmogorov-Smirnoff test. Normally distributed data were analyzed using a Student *t*-test for two groups and an ANOVA followed by a Bonferroni post hoc test for three groups or more. Data that were not normally distributed were analyzed using a Mann-Whitney test for unpaired data and, when comparing three groups or more, a Kruskal-Wallis test with a Dunn multiple comparisons post hoc test; $P < 0.05$ was considered statistically significant.

RESULTS

Laparotomy and intrauterine injection of LPS on E16 induced preterm parturition in 12.7 ± 7 h, compared to 57.5 ± 16 h ($P < 0.001$) in the surgical sham control group and 58.5 + 3.4 ($P < 0.001$) in the naive group (Fig. 1A). At labor, after intrauterine LPS, about 14.6 ± 31% of the fetuses were alive, and after PBS intrauterine injection, 83 ± 25% of the fetuses were alive ($P < 0.0001$; Fig. 1B). In naive controls, 100% of the fetuses were alive.

Leukocyte Trafficking in the Intrauterine LPS Model of Preterm Labor

At 7 h post-LPS injection, densities of the ''inflammatory'' Ly-6C^{HI} subset of monocytes in the myometrium were unchanged compared to E16 controls, whereas a substantial increase in this population was found in sham PBS-injected mice (Fig. 2A). These unexpected results were mirrored by neutrophils, where, again, levels increased considerably in sham mice compared to LPS-treated mice, although the latter were higher than in E16 untreated controls (Fig. 2D). During labor in LPS-treated mice, neutrophil cell density in the myometrium was higher compared to labor in the sham group. Although resident macrophage (CD11b+, F4/80^{HI}) densities remained relatively constant in the different groups, elevated CD86 expression suggested that these cells were activated during LPS-induced labor compared to vehicle controls (Fig. 2F).

In view of the several-fold increase in Ly-6C^{HI} cells associated with myometrial tissue at 7 h postsurgery and PBS injection, we wanted to confirm whether these represented an infiltrating blood-derived monocyte population or only a subpopulation of resident macrophages with elevated expression of the Ly-6C antigen. We performed in vivo BrdU labeling as a method to identify vascular Ly-6C^{HI} monocytes recently mobilized from the proliferating bone marrow pool [22]. BrdU was injected 24 h before harvest of tissue at the postintrauterine PBS 7-h time point. We found that ~40% of the myometrial Ly-6C^{HI} cells were BrdU positive, suggesting that these cells were derived from vascular Ly-6C^{HI} monocyte pool (Supplemental Fig. S1, A and C; Supplemental Data are available online at www.biolreprod.org). Only minor labeling of resident macrophages was observed (Supplemental Fig. S1, B and D), indicating a relatively low rate of cell proliferation in the myometrium.

The leukocyte profiles in the blood of both PBS- and LPS-injected mice 7 h postlaparotomy were indicative of systemic inflammation (Fig. 3, A–C), showing a monocytopenia and a

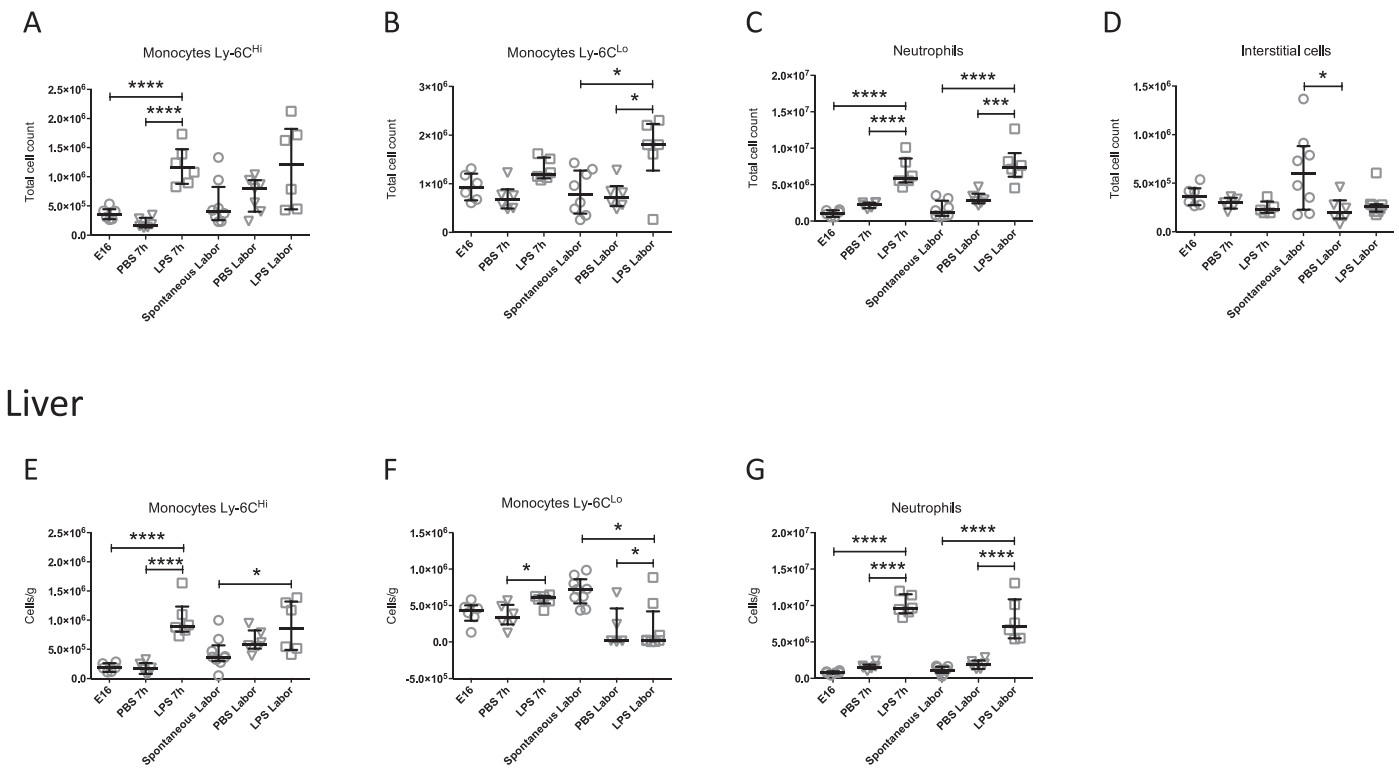

FIG. 4. Cell counts and density of leukocytes in the lungs (**A**–**D**, n = 6–8) and liver (**E**–**G**, n = 6–9) of untreated mice on E16 and following a laparotomy and intrauterine injection of either PBS or LPS (10 μg of *E. coli* LPS serotype 0111:B4) at 7 h postprocedure and in labor. Cell densities at each time point are expressed as median interquartile range. When the data were normally distributed, they were analyzed using a one-way ANOVA with Bonferroni posttest, and when the data were not normally distributed, they were analyzed using a Kruskal-Wallis test with a Dunn multiple comparison posttest (*$P <$ 0.05, ***$P < 0.001$, ****$P < 0.0001$, n = 6–12).

concomitant neutrophilia. However, levels of leukocytes in the placenta did not appear to be significantly altered by surgery with or without LPS treatment (Fig. 3, D–G). Instead, the vascular response to intrauterine LPS was most evident from the marked increase in Ly-6C^{HI} monocyte and neutrophil numbers in the lungs and liver at 7 h (Fig. 4, A–G), most likely marginated in capillaries, as previously described with systemic LPS injection [23]. Furthermore, increased expression of CD86 in interstitial lung macrophages (F4/80, MHCII+, Ly6C^{low}) suggested activation of this population in intrauterine LPS-injected mice (Supplemental Fig. S2). Therefore, although intrauterine LPS produces limited leukocyte infiltration into the uterus, it has a marked systemic effect.

The Effect of Intrauterine LPS on Local and Systemic Cytokines

In order to investigate the mechanisms controlling leukocyte trafficking in the LPS-induced PTL model, we measured the myometrial (Fig. 5, A–H) and circulating (Fig. 6, A–H) levels of chemokines and cytokine at 3 and 7 h post-LPS and during labor. Virtually all were elevated at each time point in both compartments compared to PBS controls (Figs. 5 and 6). However, this was not reflected in a greater myometrial monocyte and neutrophil infiltration, as described above, possibly due to the reduction in chemokine gradient caused by the elevated circulating levels. Laparotomy and PBS injection alone increased CCL2, IL-6, and IL-1β levels in the myometrium at 3 h (<0.0001, 0.0050, and <0.0001, respectively), but by 7 h post-PBS, chemokine and cytokine levels were comparable to naive controls (Figs. 5 and 6).

Pulmonary tissue cytokine levels were also significantly increased in the LPS-treated dams compared to PBS controls, the CCL2 specifically perhaps accounting for the increased monocyte cell numbers (Supplemental Fig. S3, A–H). The elevated inflammatory cytokines confirmed the presence of significant inflammation in lung tissue (Supplemental Fig. S3, A–D).

No Evidence of Fetal Brain Inflammation Prior to Fetal Death

In this part of the study, in mice injected with intrauterine LPS, 100% of the fetuses were alive at 3 h, 44% at 7 h, and 0% at labor; in contrast, all of the fetuses of the mice injected with intrauterine PBS were alive at each time point. Despite this, fetal brain chemokine and cytokine mRNA expression levels were generally lower in the fetuses of the mice receiving LPS at 3 and 7 h. However, during labor, when 100% of the fetuses of the mice receiving LPS were dead, levels of CCL2, CCL5, CCL20, CXCL1, and TNFα in the LPS-injected mice were found to be increased compared to PBS-injected controls (Supplemental Fig. S4, A–C).

Placental Inflammation Increases Progressively Post-Intrauterine LPS Infection

In contrast to the fetal brain samples, inflammation in the placenta, as shown by increased chemokine and cytokine

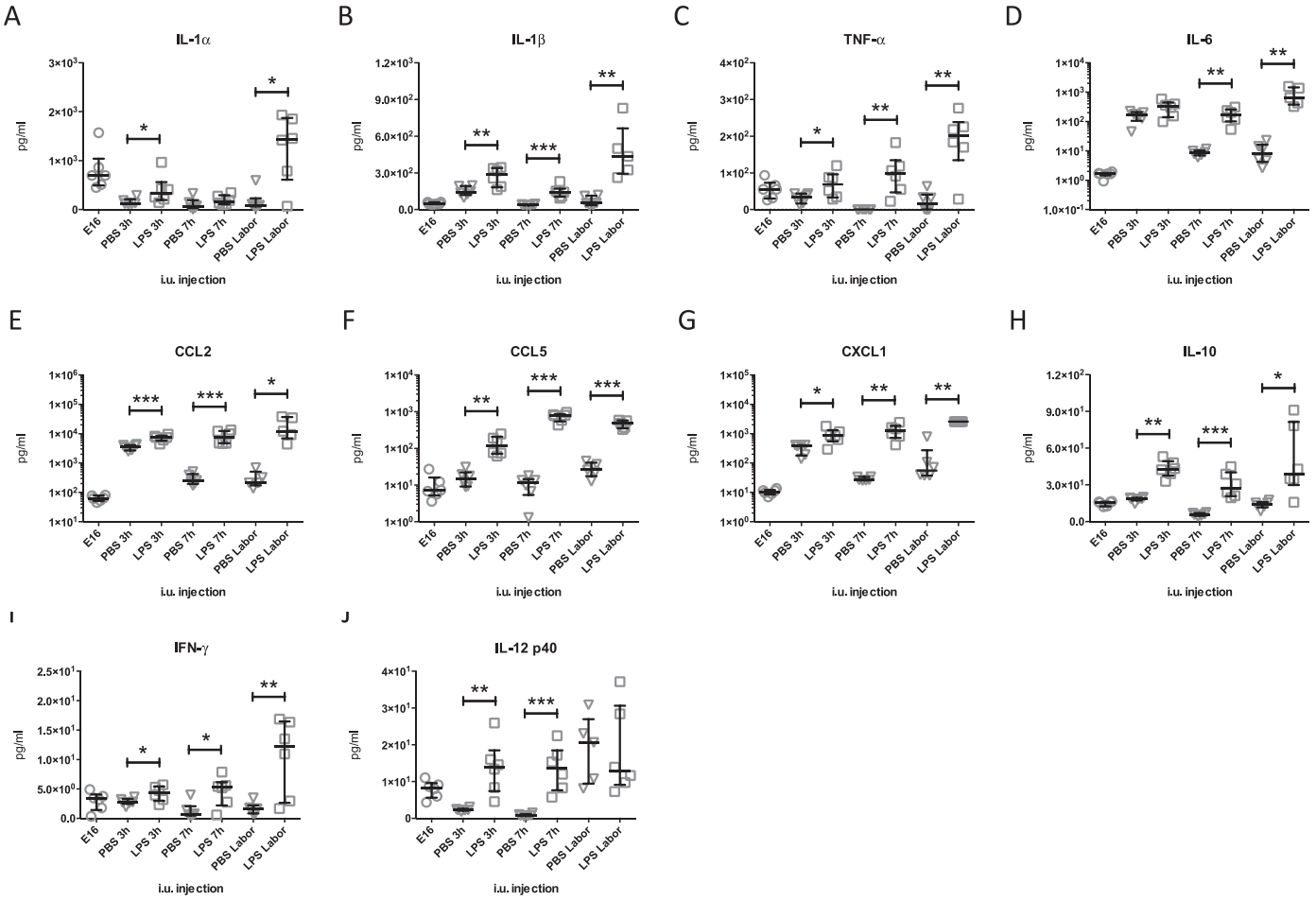

FIG. 5. **A–J**) Cytokine levels in the myometrium of untreated mice on E16 and following a laparotomy and intrauterine injection of either PBS or LPS (10 μg of *E. coli* LPS serotype 0111:B4) at 3 and 7 h postprocedure and in labor. The data are expressed as median interquartile range. Cytokine levels at each time point were compared using an unpaired *t*-test when normally distributed and a Mann-Whitney test when not. E16 untreated data were included as a guide (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, n = 5–6).

mRNA expression, increased progressively in LPS-injected mice compared to PBS controls (Supplemental Fig. S4, D–F).

Activation of Signal Transduction Pathways

After LPS injection, the MAPK/AP-1 pathway was activated, with increased phosphorylation of MEK1 and p38 at 3 h post-LPS (Fig. 7, A and E). ERK1/2 increased slightly and nonsignificantly at 3 h and then declined and was significantly lower in labor than the 3-h peak (Fig. 7C). JNK showed a progressive decline in phosphorylation (Fig. 7G). Phospho-cJun behaved in a similar fashion to ERK1/2, with a nonsignificant increase at 3 h and then a decline to a significantly lower level in labor than at the 3-h peak (Fig. 7I). In contrast to the MAPK/AP-1 system, phospho-p65 levels increase progressively, peaking in labor (Fig. 7K). In addition, at 7 h and in labor, phospho-p65 levels were higher in LPS-treated mice compared to PBS controls (Fig. 7L).

DISCUSSION

This study shows that intrauterine LPS causes marked systemic inflammation that promotes leucocyte infiltration into the lungs and liver but with relatively little myometrial or placental infiltration until the onset of parturition. This is associated with increased circulating and myometrial cytokines, the latter associated with activation of the NFκB and MAPK/AP-1 pathways. Fetal death occurred in the absence of any increased fetal brain cytokine expression prior to death but with evidence of placental inflammation.

The lower numbers of infiltrating neutrophils and Ly-6C^{HI} monocytes in the myometrium at 7 h in LPS-injected compared to PBS-treated mice did not mean less inflammation; indeed, at 3 h after LPS injection, myometrial activation of NFkB and cytokine levels were much greater than in PBS-injected mice. Similarly, systemic inflammation, demonstrated by plasma chemokine and cytokine protein levels, was also significantly increased. The high levels of cytokines in both compartments were sustained from 3 h to the onset of labor. The marked leucocyte infiltration into the lungs was associated with increased local levels of cytokines at 7 h post-intrauterine LPS challenge, suggesting that the response of the innate immune system to the intrauterine LPS may be associated with significant lung damage. Our group has previously shown that a subclinical endotoxemia is sufficient to induce monocyte mobilization from the bone marrow and a prolonged

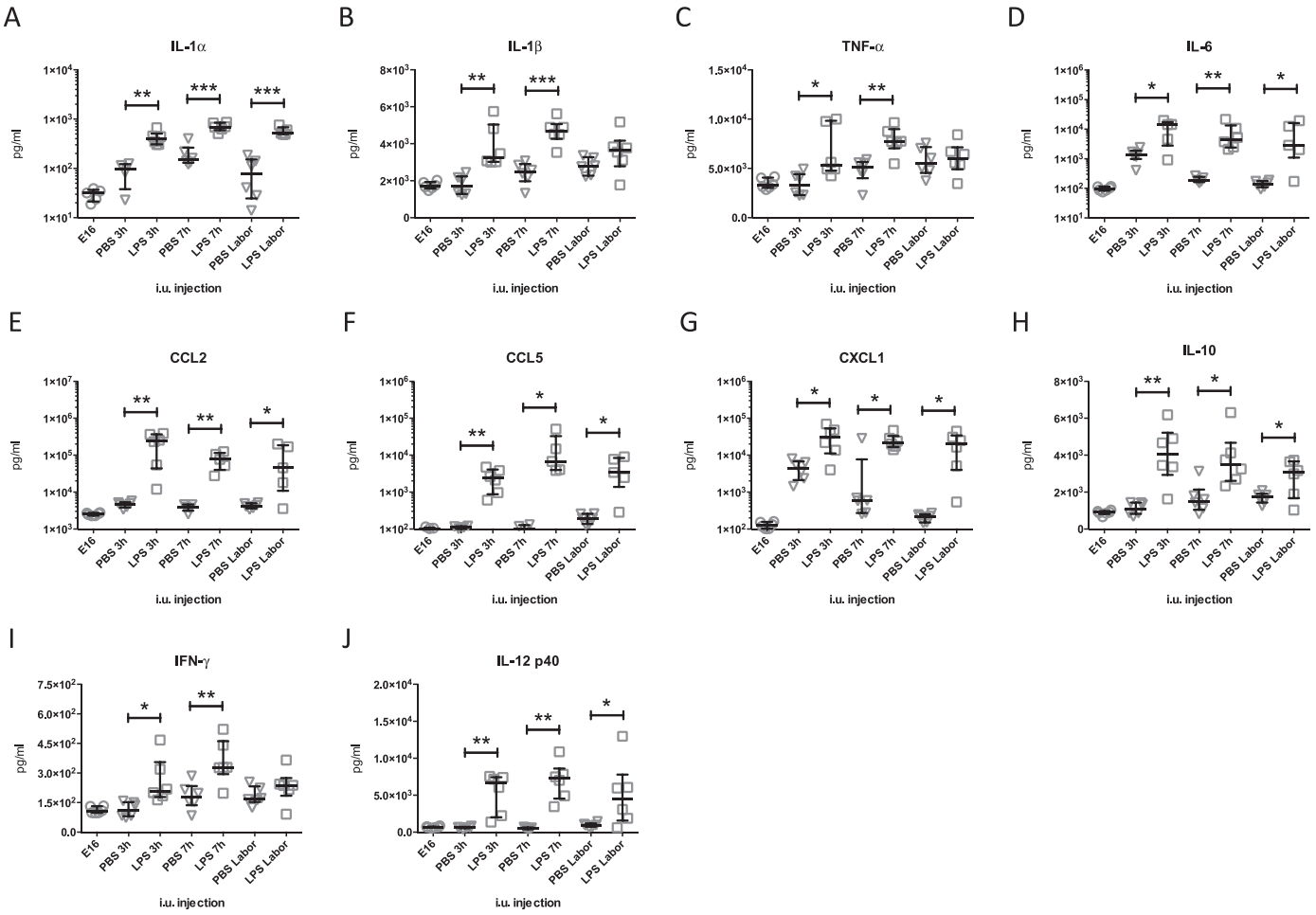

FIG. 6. **A–J**) Circulating cytokine levels of untreated mice on E16 and following a laparotomy and intrauterine injection of either PBS or LPS (10 μg of *E. coli* LPS serotype 0111:B4) at 3 and 7 h postprocedure and in labor. The data are expressed as median interquartile range. Cytokine levels at each time point were compared using an unpaired *t*-test when normally distributed and a Mann-Whitney test when not. E16 untreated data were included as a guide (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; n = 4–6).

margination of these monocytes in the lung microvasculature [24]. Further, O'Dea et al. [23] demonstrated that subclinical endotoxemia was able to prime the inflammatory marginated monocytes to a second LPS challenge. The high numbers of leukocytes sequestered to the maternal lungs and liver at 7 h post-LPS may be a limiting factor in their recruitment to the myometrium. This has been previously reported by Andonegui et al. [25], who demonstrated that when neutrophils are sequestered in the lungs, they are unavailable for entry into other sites of infection. A higher dose of LPS would be expected to increase inflammatory cell mobilization from the bone marrow, but it would also have a greater effect on the margination of inflammatory cells into the lung vasculature. Overall, it is difficult to predict whether the net effect would be to increase the myometrial inflammatory cell infiltration. Indeed, the neutrophil and monocyte cell density in the myometrium was significantly higher after PBS injection than LPS. This may have been because of the greater systemic inflammation, as shown by the higher levels of circulating chemokines and cytokines at all time points, and inflammatory cell sequestration into the lungs and liver after LPS, but it is intriguing that the markedly greater myometrial inflammatory cell infiltration in the PBS group (compared with control or LPS-injected mice) was not associated with an earlier onset of labor. This—and the relatively lesser increases (compared to the 7-h PBS levels) in monocyte and neutrophil numbers in LPS-induced laboring myometrium—suggests that intrauterine LPS causes labor through a direct effect on myometrial function and/or mediated via a TLR4-driven increase in local inflammation, as has been suggested by in vitro studies [26].

Macrophages may also be a target of LPS action, as they are present in the uterus and express TLR4 [27]. Our earlier work showed that inflammatory cytokine levels were increased at 2 h post-LPS in association with increased COX-2 mRNA expression at 4 h [16]. In the current study, we found that myometrial cytokines were increased at 3 and 7 h and in labor, consistent with these observations. Shynlova et al. [17] demonstrated that administration of a broad-spectrum chemokine inhibitor significantly delayed LPS-induced PTL and also reduced the LPS-induced increase in IL-1β, IL-6, IL-12, CSF2, CCL2, CCL4, CXCL1, and CXCL2 gene expression in the myometrium, suggesting that inflammatory cell infiltration was important in the process of labor induction. However, we previously found that IL-8 alters gene expression in human

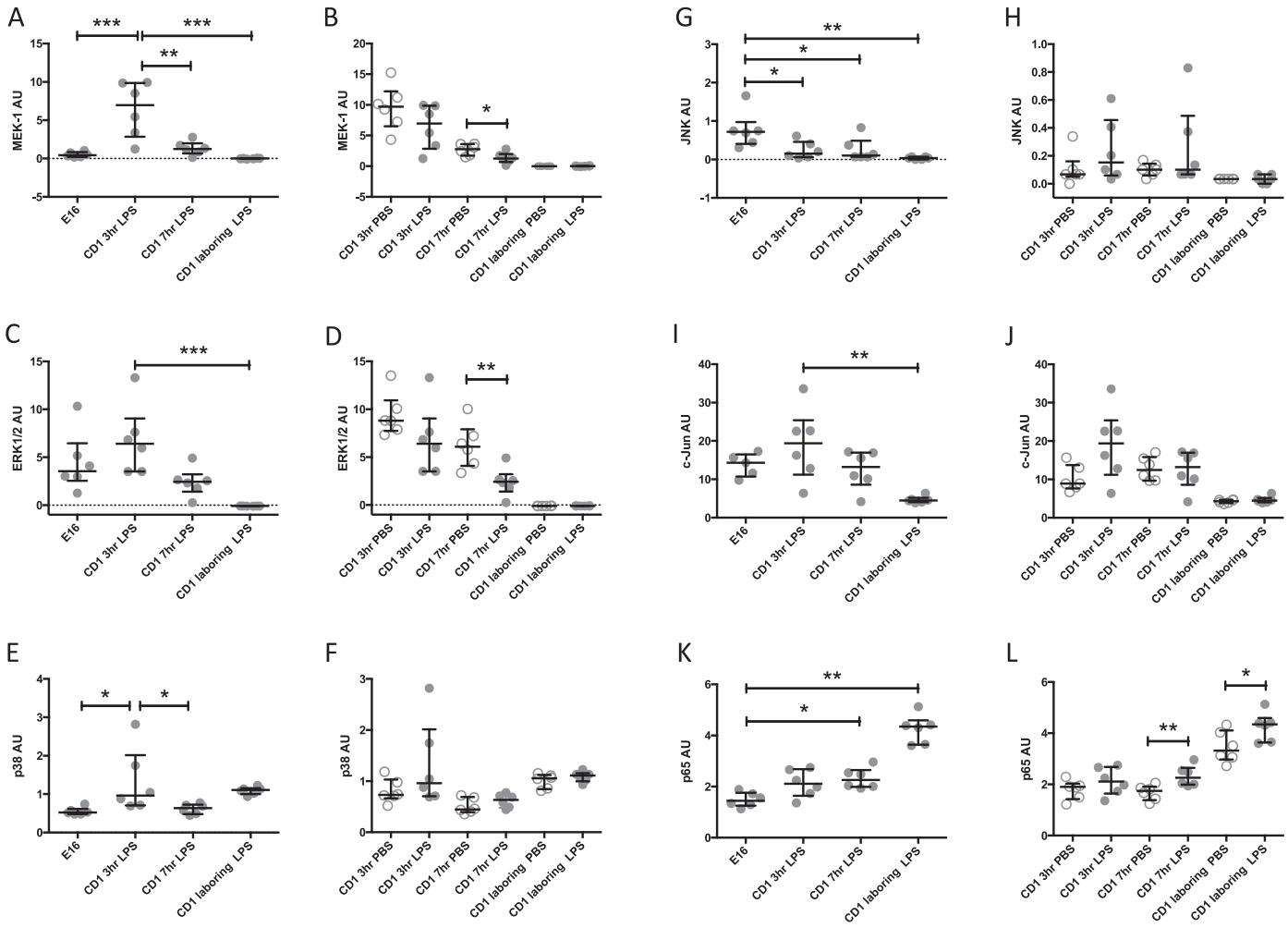

FIG. 7. MAPK phosphorylation levels (**A–F**) and MAPK/AP-1 and NFκB phosphorylation (**G–L**) in the myometrium following a laparotomy and intrauterine injection of either PBS or LPS (10 μg of *E. coli* LPS serotype 0111:B4). The data are expressed as median interquartile range. The data in graphs **A**, **C**, **E**, **G**, **I**, and **K** were analyzed using a one-way ANOVA with Bonferroni posttest when normally distributed and a Friedman test and with a Dunn multiple comparisons post hoc test when not. Data in graphs **B**, **D**, **F**, **H**, **J**, and **L** representing the levels of PBS versus LPS at each time point were analyzed using an unpaired *t*-test when normally distributed and a Mann-Whitney test when not (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; $n = 6$).

myometrial cells directly, increasing COX-2 and its own mRNA expression; consequently, a chemokine antagonist might mediate its effects independent of any reduction in inflammatory cell infiltration [28]. Moreover, others have shown that depletion of both circulating neutrophils and Ly-6C^{HI} monocytes did not delay LPS-induced PTL or term labor, respectively [14, 29], although depletion of macrophages (treating mice with anti-F4/80 antibodies) prevented preterm labor in LPS-treated mice [30]. Macrophages are the most abundant leucocyte population in the mouse myometrium and increase with advancing gestation [6]. To assess macrophage activity, we measured the expression of CD86, a costimulatory molecule found on the surface of antigen presenting cells, which correlates with an increase in proinflammatory cytokine production. In our study, CD86 expression on myometrial macrophages increased approximately threefold in LPS-induced labor versus PBS labor, but no increase was seen prior to labor at the 7-h time point. However, macrophage depletion prevents LPS-induced parturition [30], suggesting that these cells play an important role in the process, although no data showing a reduction in macrophage numbers in the uterus or cervix were presented. Isolating the infiltrating and resident populations to assess their functional and phenotypic characteristics at various stages of pregnancy may provide further insight into this area.

Different serotypes of LPS have been used in the intrauterine LPS mouse model of PTL, and this may account for the inconsistent results. For example, fetal death after an LPS challenge has been attributed to fetal brain inflammation [31], but in the current study, we found no evidence of fetal brain inflammation prior to death. Earlier work from our own group [31] and from Elovitz et al. [32] found that intrauterine LPS results in fetal brain inflammation. However, more recent data from our group indicate that these differences may be related to the different types of LPS used [19]. While Elovitz et al. [32] used the *E. coli*-derived LPS serotype O55 [32], Pirianov et al. [31] used a salmonella-derived LPS, which has major differences in both the lipid A and the oligosaccharide-specific chain of the molecule and which may alter placental transfer and therefore the fetal inflammatory response. Consistent with this, in a rat study using LPS 0111 labeled with I125, none was detected in the fetus, suggesting that LPS

TABLE 1. Primer pair sequences, GenBank/EMBL accession numbers, nucleotide numbers, and Ct values.

Name	Forward (F) and reverse (R) primer sequences (5′–3′)	GenBank/EMBL accession no.	Nucleotide no.	Ct value
GAPDH	F: 5′ACTCCACTCACGGCAAATTC-3′ R: 5′TCTCCATGGTGGTGAAGACA-3′	NM_001001303	201–371	17.52 ± 2.89
CCL2	F: 5′CCCACTCACCTGCTGCTACT-3′ R: 5′TCTGGACCCATTCCTTCTTG-3′	NM_011333	81–481	18.20 ± 1.69
CCL5	F: 5′CCCTCACCATCATCCTCACT-3′ R: 5′CCTTCGAGTGACAAACACGA-3′	NM_0.13653	51–321	17.84 ± 2.38
CXCL1	F: 5′GCCTATCGCCAATGAGCTG-3′ R: 5′AAGGGAGCTTCAGGGTCAAG-3′	NM_008176	71–321	18.10 ± 1.26
CCL20	F: 5′CGACTGTTGCCTCTCGTACA-3′ R: 5′CACCCAGTTCTGCTTTGGAT-3′	NM_016960	59–320	19.84 ± 2.45
CXCL5	F: 5′CGCTAATTTGGAGGTGATCC-3′ R: 5′GTGCATTCCGCTTAGCTTTC-3′	NM_009141	161–490	20.56 ± 1.09
IL-1β	F: 5′-CAGGCAGGCAGTATCACTCA-3′ R: 5′-AGCTCATATGGGTCCGACAG-3′	NM_008361	248–496	18.56 ± 2.23
TNF-α	F: 5′-TATGGCTCAGGGTCCAACTC-3′ R: 5′-CTCCCTTTGCAGAACTCAGG-3′	NM_013693	984–1157	17.45 ± 1.27
IL-6	F: 5′-AGTTGCCTTCTTGGGACTGA-3′ R: 5′-TCCACGATTTCCCAGAGAAC-3′	DQ_788722	33–191	18.50 ± 1.77

0111 does not cross the placenta [33]. Rather, studies in rats and sheep suggest that LPS induces neonatal brain damage and causes death through impaired placental perfusion [34, 35]. Our findings are consistent with this, although the cytokine mRNA expression in the placenta was increased at 3 and 7 h and markedly elevated in the laboring samples. In contrast, placental leucocyte infiltration of neutrophils and Ly-6C^{HI} monocytes was unchanged from controls at 7 h, while at labor, Ly-6C^{HI} monocyte cell density was actually lower in the LPS-treated mice despite the increase in cytokine mRNA expression. If there is an abrupt cessation of blood flow to the placenta, as has been suggested [34, 35], then this may lead to an altered pattern of immune response compared to the situation in the human, where chorioamnionitis is likely to develop gradually and where placental blood flow does not appear to be compromised. These are potentially notable limitations to the ability of intrauterine LPS to reproduce the changes seen with chorioamnionitis in human pregnancies. However, a substantial literature suggests that inflammation plays a role in the onset of human term labor through upregulation of prolabor genes [36], repression of progesterone action [37], or a direct effect on myometrial contractility [26, 38]. Indeed, we previously found commonalities between LPS-induced labor in the mouse and human labor [39], suggesting that intrauterine LPS may be a reasonable model for human term labor.

In this study using a murine model of intrauterine LPS-induced PTL, we found no evidence of a relationship between labor onset or fetal demise and changes in leucocyte populations in reproductive tissues and systemic sites. Generally, this model is used to reproduce the changes seen with chorioamnionitis in human pregnancies, and while it does reliably induce labor, our data suggest that this may be through an increase in myometrial cytokine levels or a direct effect of LPS on myometrial function rather than an increase in myometrial inflammatory cell infiltration. Although this mechanism is probably active in human chorioamnionitis, it seems likely that inflammatory cells also have an important role in the onset of chorioamnionitis-associated preterm labor, meaning that the murine LPS model does not totally reproduce all of the aspects of human chorioamnionitis [38]. Further, in this study, despite placental inflammation, fetal death occurred without evidence of preceding fetal inflammation and may have been caused by a reduction in placental perfusion [40]. In contrast, in the human, inflammation plays a prominent role in the fetal compromise [41, 42]. Overall, our data suggest that the intrauterine LPS mouse model reproduces some but not all of the features of human chorioamnionitis.

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