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Chronic Fetal Exposure to *Ureaplasma parvum* Suppresses Innate Immune Responses in Sheep

Suhas G. Kallapur,*†‡ Boris W. Kramer,§ Christine L. Knox,¶ Clare A. Berry,† Jennifer J. P. Collins,§ Matthew W. Kemp,‖ Ilias Nitsos,‡ Graeme R. Polglase,† James Robinson,¶ Noah H. Hillman,*†‡ John P. Newnham,† Claire Chougnet,*‖ and Alan H. Jobe*†‡

The chorioamnionitis associated with preterm delivery is often polymicrobial with ureaplasma being the most common isolate. To evaluate interactions between the different proinflammatory mediators, we hypothesized that ureaplasma exposure would increase fetal responsiveness to LPS. Fetal sheep were given intra-amniotic (IA) injections of media (control) or *Ureaplasma parvum* serovar 3 either 7 or 70 d before preterm delivery. Another group received an IA injection of *Escherichia coli* LPS 2 d prior to delivery. To test for interactions, IA *U. parvum*-exposed animals were challenged with IA LPS and delivered 2 d later. All animals were delivered at 124 ± 1-d gestation (term = 150 d). Compared with the 2-d LPS exposure group, the *U. parvum* 70 d + LPS group had 1) decreased lung pro- and anti-inflammatory cytokine expression and 2) fewer CD3* T lymphocytes, CCL2*, myeloperoxidase*, and PU.1* cells in the lung. Interestingly, exposure to *U. parvum* for 7 d did not change responses to a subsequent IA LPS challenge, and exposure to IA *U. parvum* alone induced mild lung inflammation. Exposure to *U. parvum* increased pulmonary TGF-β1 expression but did not change mRNA expression of either the receptor TLR4 or some of the downstream mediators in the lung. Monocytes from fetal blood and lung isolated from *U. parvum* 70 d + LPS but not *U. parvum* 7 d + LPS animals had decreased in vitro responsiveness to LPS. These results are consistent with the novel finding of downregulation of LPS responses by chronic but not acute fetal exposures to *U. parvum*. The findings increase our understanding of how chorioamnionitis-exposed preterm infants may respond to lung injury and postnatal nosocomial infections.


Preterm births account for 12% of all deliveries in the United States and are the most important determinant of neonatal mortality and morbidity (1). Chorioamnionitis or inflammation in the fetal membranes is associated with ∼60% of deliveries before 30 wk of gestation (2). The most common organisms isolated from the amniotic fluid of women with chorioamnionitis are the *Ureaplasma* species (3). Furthermore, among the preterm infants with chorioamnionitis caused by ureaplasma species, ∼60% also had coinfection with other organisms (3). In addition, ureaplasmas were isolated from amniotic fluid from first or second trimester or from washed semen in assisted reproduction (4–6). Therefore, preterm infants have frequent and often prolonged exposure to Ureaplasma. Ureaplasma colonization of the upper genital tract can induce preterm labor in nonhuman primates and is associated with preterm labor in humans (5, 7). Exposure of preterm infants to *Ureaplasma* is associated with increased risk for adverse pulmonary, gastrointestinal, and neurologic outcomes (7–10).

The two species of ureaplasmas, which colonize humans, are *Ureaplasma urealyticum* (serovars 2, 4, 5, 7, and 13) and *Ureaplasma parvum* (serovars 1, 3, 6, and 14) (8). Of these, *U. parvum* is the most common species isolated from preterm neonates and upper genital tracts of women (11, 12). The ureaplasmas are unusual bacteria in that they have a plasma membrane but lack a peptidoglycan cell wall, use urea as the sole source of energy, and are dependent on the host for other metabolic functions (13).

We have reported acute and chronic exposure to ureaplasma species in fetal sheep (14, 15). Intra-amniotic (IA) injection of *U. parvum* in sheep induces a robust colonization of fetal chorioamnion and lung with poor bacterial clearance (14). Exposure to *U. parvum* in sheep induces a mild fetal inflammatory response, increases pulmonary surfactant pools, and results in mild transient developmental abnormalities in the lung (16, 17). In contrast, IA injection of LPS induces a robust fetal inflammation in the sheep (18). Interestingly, repeated exposures in vivo to IA LPS induce endotoxin tolerance and cross-tolerance to other Toll-like agonists (19, 20). *Ureaplasma* species signal via TLR1/2/6 and can increase LPS-mediated inflammation in vitro (21, 22). Although coinfection of *Ureaplasma* spp with other organisms is common in chorioamnionitis (3), the interactions between ureaplasma and LPS in the fetus have not been explored.

On the basis of the in vitro observation that ureaplasma increase LPS-mediated inflammation (21, 22), we hypothesized that exposure to ureaplasma would increase LPS induced fetal inflammation. Pregnant ewes were given an IA injection of *U. parvum* serovar 3 to induce acute or chronic chorioamnionitis. We subsequently...
challenged fetal sheep with an IA injection of LPS 2 d prior to preterm delivery at 82% of term gestation. Fetal inflammation was assessed after a single exposure to *U. parvum* or LPS or after combined exposures.

**Materials and Methods**

### Animals and treatments

The animals were studied in Western Australia with approval from the animal care and use committees of the Cincinnati Children’s Hospital (Cincinnati, OH) and the University of Western Australia. Time-mated Merino ewes with singleton fetuses were randomly assigned to study groups of five to seven animals (Table I). The pregnant sheep were given ultrasound-guided IA injections of 1) *U. parvum* seorov 3 (U. parvum), 2 × 10^7 CFU or 2 ml media (control) into the amniotic fluid 70 or 7 d prior to delivery, 2) *E. coli* LPS (OSS:BS; Sigma-Aldrich, St. Louis, MO) 10 mg in 2 ml saline 2 d prior to delivery, or saline only (control). To evaluate immune modulatory effects of *U. parvum* on LPS, separate groups of animals received IA LPS 2 d before delivery after exposure to IA *U. parvum* 70 or 7 d before delivery. All fetal injections were given with ultrasound guidance and with electrolyte analysis to confirm injection into amniotic fluid (23). All animals were delivered at 124 ± 1 d gestational age, umbilical cord blood was collected for plasma and for circulating leukocytes and fetuses were given lethal intravenous doses of pentobarbital for euthanasia. At autopsy tissue from the right lower lobe of the lung and live lung tissues were snap frozen for RNA extraction. For bronchoalveolar lavage fluid (BALF), the left lung was inflated with normal saline to total lung capacity followed by withdrawal, and the procedure was repeated three times (23). BALF were pooled and used for cell counts and protein measurements (24). BALF cell counts were expressed as total cells recovered from the lavage normalized to body weight. Lung compliance was measured from the deflation ratio of an air pressure–volume curve with the chest open (24). The right upper lobe of the lung was inflated fixed with 10% buffered formalin at 30 cm H2O pressure for morphology.

### Culture of lung and blood monocytes

After exsanguination of the fetus, the lung was chopped thoroughly into fine pieces and incubated in RPMI 1640 medium (25). The lung suspension was then gently passed through a 100-μm mesh filter and the suspension was washed twice with PBS. Cells from the suspension were then layered over discontinuous Percoll gradients (1.085 and 1.046 g/ml) (Amersham Bioscience, Piscataway, NJ) to separate the mononuclear cells from the red blood cells at the interface between the Percoll densities (25). Whole blood diluted 1:1 with saline was layered on a 1.046 g/ml Percoll gradient to recover mononuclear cells. Cells were counted using trypan blue to evaluate viability and then plated in culture dishes using media supplemented with 10% heat-inactivated FCS (Sigma-Aldrich, Castle Hill, NSW, Australia). After incubation at 37˚C for 2 h, nonadherent cells were removed, and plates were washed twice with PBS. Monocytes were challenged with media only or LPS (100 ng/ml) for 16 h (19). Cell responsiveness as indicated by IL-6 secretion was measured in the media with a sandwich ELISA (coating Ab – mouse anti-ovine IL-6 (number MAB10445; Chemicon), and primary Ab guinea pig anti-ovine IL-6 (number AB1840; Chemicon)), and MCP-1 (rabbit anti-ovine MCP-1 coating Ab, and primary Ab guinea pig anti-ovine MCP-1 detection Ab (Seven Hills Bioreagents)). The detection Ab in all the assays was an appropriate species-specific HRP-conjugated Ab. Plasma haptoglobin was measured by ELISA (anti-bovine kit; ICL, Newberg, OR).

### Data analysis

Results are given as mean ± SEM. Comparisons among three or more groups were performed by analyses of variance with Student-Newman-Keuls tests used for post hoc analyses. Comparison of two groups was done by a nonparametric t test (Mann–Whitney U test) for data not distributed normally and Student t test for normally distributed data. Statistical significance was accepted at p < 0.05.

### Results

All animals given an IA injection of *U. parvum* had positive cultures for *U. parvum* in the amniotic fluid (Table I). The amniotic fluid *U. parvum* titer tended to be lower (statistically not significant) after a 7-d exposure compared with 70-d exposure, but there was variability within each group. *U. parvum* did not cause gross developmental abnormalities and the birth weights were similar between infected and uninfected control fetuses (Table I). Consistent with our previous reports (15), compared with controls, the lung gas volumes increased ∼3-fold after chronic but not acute *U. parvum* exposure (Table I) indicating lung maturation. The 2-d LPS group had increased lung weight relative to body weight but decreased lung volume relative to lung weight. Low-power lung histology demonstrated that the control and the *U. parvum* only-exposed animals (both 7 and 70 d) had a similar histology (Fig. 1A–C). In contrast, 2-d LPS exposure recruited inflammatory cells in the airways and lung interstitium (Fig. 1D). The histology in animals exposed to acute *U. parvum* 7 + 2 d LPS was indistinguishable from the 2-d LPS only (compare Fig. 1D, 1E), whereas chronic *U. parvum* 70 + 2 d LPS-exposed animals had a lung histology similar to controls (compare Fig. 1F, 1A).

### Downregulation of LPS induced recruitment of leukocytes in the lung by prior chronic exposure to *U. parvum*

Control fetal lambs had very few neutrophils or monocytes in the pulmonary airspaces (Fig. 2A, 2B). Exposure to IA *U. parvum* for 7 d caused no significant recruitment of neutrophils, whereas a chronic exposure (70 d) recruited qualitatively higher numbers of neutrophils and monocytes to the airspaces. IA LPS induced a large recruitment of neutrophils and monocytes. Fetal lambs...
exposed to either LPS alone or *U. parvum* 7 d + LPS had similar numbers of inflammatory cells in the airspaces. However, animals exposed to *U. parvum* 70 d + LPS had qualitatively fewer inflammatory cells in the pulmonary airspaces.

Very few lymphocytes were detected in the airspaces of animals from any of the groups (data not shown). We therefore evaluated recruitment of CD3<sup>+</sup> T lymphocytes in the fetal lung. Control and *U. parvum*-exposed fetal lambs had a few T lymphocytes in the lung (Fig. 2C, 2E, 2G). Exposure to IA LPS recruited T lymphocytes in the lung, and this recruitment was almost completely blocked by a prior chronic but not acute exposure to *U. parvum* (compare Fig. 2F, 2H). PU.1 is an ets-domain developmental transcription factor that orchestrates the inflammatory processes in myeloid and nonmyeloid cells (25, 28, 29). A developmental transcription factor that orchestrates the inflammatory cytokines IL-1β (4-fold), IL-6 (2-fold), IL-8 (7-fold), serum amyloid A3 (17-fold), and IL-1 receptor antagonist (IL-1ra) (4-fold) expression in the lung. In contrast, exposure to IA LPS for 2 d greatly increased expression of both the proinflammatory cytokines/chemokines (IL-1β, IL-6, IL-8, MCP-1 [CCL2], serum amyloid A3, CXCL9, and CXCL10), and the anti-inflammatory cytokines IL-1RA and IL-10 in the lung. Prior exposure to *U. parvum* caused an inconsistent or modest increase in expression of IL-1β (4-fold), IL-6 (2-fold), IL-8 (7-fold), serum amyloid A3 (17-fold), and IL-1 receptor antagonist (IL-1ra) (4-fold) expression in the lung. In contrast, exposure to IA LPS for 2 d greatly increased expression of both the proinflammatory cytokines/chemokines (IL-1β, IL-6, IL-8, MCP-1 [CCL2], serum amyloid A3, CXCL9, and CXCL10), and the anti-inflammatory cytokines IL-1RA and IL-10 in the lung. Prior exposure to *U. parvum* caused an inconsistent or modest increase in expression of IL-1β (4-fold), IL-6 (2-fold), IL-8 (7-fold), serum amyloid A3 (17-fold), and IL-1 receptor antagonist (IL-1ra) (4-fold).

### Table I. *Ureaplasma* titers and lung volumes in fetal lambs after IA exposures

<table>
<thead>
<tr>
<th>Groups</th>
<th>Titer (CFU × 10&lt;sup&gt;6&lt;/sup&gt;/ml)</th>
<th>Lung/Body Weight (g/kg)</th>
<th>V&lt;sub&gt;40&lt;/sub&gt; ml/g Lung Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0 (0, 0)</td>
<td>31.3 ± 0.4</td>
<td>0.31 ± 0.01</td>
</tr>
<tr>
<td>7 d UP</td>
<td>4.2 (3.6, 75.3)</td>
<td>34.6 ± 2.1</td>
<td>0.36 ± 0.05</td>
</tr>
<tr>
<td>70 d UP</td>
<td>10.2 (6.8, 128.9)</td>
<td>33.0 ± 2.2</td>
<td>0.98 ± 0.19</td>
</tr>
<tr>
<td>2 d LPS</td>
<td>2.7 ± 0.1</td>
<td>36.8 ± 2.1*</td>
<td>0.18 ± 0.02*</td>
</tr>
<tr>
<td>7 d UP + 2 d LPS</td>
<td>0.4 (0.07, 1.7)</td>
<td>37.3 ± 1.0*</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td>70 d UP + 2 d LPS</td>
<td>6.1 (1.2, 39.6)</td>
<td>36.1 ± 1.2*</td>
<td>0.90 ± 0.19*</td>
</tr>
</tbody>
</table>

Lung, birth weight, and V<sub>40</sub> means ± SEM. *U. parvum* titer, median (25th percentile, 75th percentile). All animals delivered at 124 ± 1-d gestation (term = 150 d). V<sub>40</sub>, lung volume at 40 cm H<sub>2</sub>O pressure.

* <i>p</i> < 0.05 versus controls.

### In vitro responses of lung and blood monocytes to LPS

Exposure to chronic *U. parvum* could decrease LPS responsiveness via secretion of a soluble inhibitor or could induce changes in cell responsiveness to LPS. To evaluate these possibilities, monocytes from both the lung and the peripheral blood were cultured and challenged with LPS in vitro. Consistent with immature LPS responsiveness, fetal monocytes from control lambs had a decreased response to LPS compared with blood monocytes and alveolar macrophages from adult ewes to an in vitro LPS challenge (increase in IL-6 secretion: 2-fold versus 16-fold for blood monocytes and 2- versus 9-fold for lung monocytes; <i>p</i> < 0.05) (Fig. 3A, 3B). Exposure to *U. parvum* alone did not increase responsiveness to LPS in vitro. However, monocytes from fetal lambs exposed to IA LPS increased IL-6 secretion in response to LPS challenge in vitro, indicating a maturation effect. Interestingly, monocytes from IA LPS + *U. parvum* (7 d) further increased IL-6 secretion in response to an in vitro LPS challenge compared with the 2-d LPS group and similar to levels from adult cells. In striking contrast, monocytes from the IA LPS + *U. parvum* 70-d group had a blunted response to LPS challenge in vitro.

### Downregulation of LPS induced cytokine expression in the lung by prior chronic exposure to *U. parvum*

We previously demonstrated that the fetal lung is a target of inflammation after exposure to IA administration of proinflammatory agonists (24). We therefore measured expression of proinflammatory cytokine, chemokine, acute phase reactant and anti-inflammatory cytokine mRNAs in the fetal lung (Fig. 4). Relative to controls, either acute (7-d exposure) or chronic (70-d exposure) to *U. parvum* caused an inconsistent or modest increase in expression of IL-1β (4-fold), IL-6 (2-fold), IL-8 (7-fold), serum amyloid A3 (17-fold), and IL-1 receptor antagonist (IL-1ra) (4-fold) expression in the lung. In contrast, exposure to IA LPS for 2 d greatly increased expression of both the proinflammatory cytokines/chemokines (IL-1β, IL-6, IL-8, MCP-1 [CCL2], serum amyloid A3, CXCL9, and CXCL10), and the anti-inflammatory cytokines IL-1RA and IL-10 in the lung. Prior exposure to *U. parvum* for 7 d did not change responses to LPS. In sharp contrast, chronic exposure to *U. parvum* significantly downregulated LPS responses to near control levels. To further confirm these findings, we measured IL-1β and IL-8 protein in the BALF. Chronic exposure to *U. parvum* alone modestly increased secretion of IL-8 in the lung (Fig. 5B). Neither acute nor chronic exposure to *U. parvum* induced pulmonary IL-1β secretion (Fig. 5A). Consistent with the mRNA responses, IA LPS greatly increased IL-1β and IL-8 secretion (Fig. 5). Similar to the mRNA effects, chronic but not acute prior exposure to *U. parvum* decreased LPS-mediated induction of IL-1β and IL-8 secretion.

**FIGURE 1.** Lung histology after intraamniotic exposure to LPS, acute, and chronic IA exposure to *U. parvum*. Representative photomicrographs showing H&E staining of lung sections from the animals with intraamniotic exposures to controls (A), 7-d *U. parvum* (B), 70-d *U. parvum* (C), 2-d LPS (D), 7-d *U. parvum* + 2-d LPS (E), and 70-d *U. parvum* +2-d LPS (F). Note the similarity of histology in A, B, C, and F; D and E demonstrate inflammatory cells in the airways and lung interstitium. Scale bar, 100 μm.
Downregulation of LPS induced activation of inflammatory cells in the lung by prior chronic exposure to U. parvum

MCP-1 and MPO are expressed by activated neutrophils and monocytes in fetal lambs (27, 30). IA LPS induced expression of MCP-1 predominantly in the inflammatory cells with some expression in the pulmonary epithelial cells (Fig. 6). Consistent with the cytokine expression, U. parvum (either acute or chronic exposure) did not increase MCP-1 expressing cells in the lung. Prior acute exposure to U. parvum did not modify LPS effects, but strikingly, chronic exposure to U. parvum significantly reduced LPS-induced increases in MCP-1 expressing cells. Similar to MCP-1, U. parvum also did not increase MPO expressing cells in the lung (Fig. 7). Prior chronic but not acute exposure to U. parvum decreased LPS induced increasing in MPO+ cells in the lung.

Systemic acute phase response to LPS was not inhibited by prior chronic exposure to U. parvum

Next, we asked whether U. parvum modulated IA LPS induced systemic fetal inflammation. We measured the expression of acute phase response genes Serum amyloid A3 and CRP in the fetal liver (Fig. 8A, 8B) and haptoglobin levels in the plasma (Fig. 8C). The expression of serum amyloid A3, CRP and plasma haptoglobin were low in control lambs and those exposed to acute U.parvum had low levels of TGF-β1 expression in the airways (Fig. 9A). Chronic U. parvum, LPS, or acute U. parvum + LPS exposure equivalently increased TGF-β1 secretion. Interestingly, the group chronic U. parvum + LPS had the highest levels of BALF TGF-β1. Next, we quantified expression of several TLR4 signaling molecules. IL-1 receptor associated kinases (IRAKs) 1 and 4 are positive regulators, whereas IRAK-M is a negative regulator.
of TLR4 signaling (32, 33). TLR4 and IRAK-M mRNA expression increased 2.5- to 3-fold in the fetal lung exposed to IA LPS or IA LPS + *U. parvum* (7 d) (Fig. 9B, 9E), whereas the expression of IRAK-1 and IRAK-4 mRNA were similar to controls (Fig. 9C, 9D). Compared with controls, chronic exposure to *U. parvum* or *U. parvum* + LPS did not change expression of TLR4, IRAK-1, IRAK-4, or IRAK-M mRNAs in the fetal lung.

**Discussion**

We report a profound decrease in responsiveness to LPS after a chronic exposure to *U. parvum*. Expression of all the measured genes with the sole exception of TGF-β1 did not increase in the lung in response to LPS in fetal lambs chronically exposed to *U. parvum*. Consistent with previous reports (21), we observed augmentation of LPS responses in vitro in monocytes from the acute *U. parvum* + LPS group compared with the LPS group or controls. However, LPS responsiveness was blunted in monocytes from the chronic *U. parvum* + LPS group. Endotoxin tolerance is now understood to be a complex reprogramming of inflammatory and noninflammatory cells to repeated exposures to bacterial products (34). Although a large body of literature on endotoxin tolerance is derived from in vitro studies, historical studies have reported endotoxin tolerance in patients recovering from typhoid fever (35). Also, leukocytes from patients with sepsis display endotoxin tolerance (36). Our findings differ from these observations in several respects: Similar to human chorioamnionitis, IA injection of *U. parvum* causes local colonization of the organism in the organs contacting the amniotic fluid (e.g., lung, chorioamnion, gut, and the skin) but infrequently causes systemic bacteremia (10, 14). Second, we found a near global decreased capacity to respond to...
LPS in the fetal lung after a chronic *U. parvum* exposure rather than a complex reprogramming of inflammation. Third, the downregulated LPS responsiveness was demonstrated 70 d after exposure but not after a 7-d exposure. Our study is unique in that, the host is a preterm fetus with developmental immaturity of the immune system.

Because *Ureaplasma* spp are common colonizers of the lower genital tract, there is some debate about the pathogenicity of the organism in pregnancy. However, several studies have unequivocally demonstrated the association of female upper genital *Ureaplasma* colonization with preterm deliveries, fetal inflammation and adverse neonatal outcomes (8, 9, 13). Furthermore, *Ureaplasma* species can induce inflammatory cells to produce proinflammatory cytokines via TLR2/6 in vitro (21, 22). Consistent with its low virulence, we observed a persistent colonization but low-grade lung inflammation both at 7 and 70 d after IA injection of *U. parvum*. Lung inflammation after IA *Ureaplasma* exposure is likely via direct colonization, because we previously reported a 2-log order higher *Ureaplasma* titer in the fetal lung fluid compared with the amniotic fluid (14). *Ureaplasma* species are known to produce biofilms, which could trap inflammatory products thereby mechanically block inflammatory signaling (37). However, this is an unlikely explanation for the profound hypo-responsiveness to LPS in our experiments, because LPS responses were preserved when *U. parvum* exposure was for 7 d. Furthermore, both lung and blood monocytes from fetal lambs exposed to *U. parvum* for 70 but not 7 d demonstrated decreased responsiveness to LPS in vitro. Whether these responses are dependent on the degree of prematurity is not known. Also, the precise mechanism(s) by which a chronic exposure to *U. parvum* can decrease LPS responses remain to be identified.

Exposure to chronic but not acute *U. parvum* infection decreased both the influx of pulmonary inflammatory cells and expression of activation markers induced by IA LPS. Therefore, a question is whether the decreased expression of activation markers reflected decreased inflammatory cells in the fetal lung. The inflammatory cell composition in the lung 2 d after IA LPS is ∼60% neutrophils and 35% monocytes with few lymphocytes. Compared with effects of LPS alone, a prior exposure to chronic *U. parvum* reduced the neutrophil influx by ∼30% and monocytes by 60% (these reductions were not statistically significant). In contrast, the reductions in expression of cytokines, PU.1, MCP-1, and MPO+ cells were much larger, and the monocytes from fetal lambs with...
chronic *U. parvum* + LPS were poorly responsive to an in vitro LPS challenge. Taken together, these findings suggest that chronic exposure to *U. parvum* decreased activation and possibly recruitment of leukocytes to the fetal lung in response to LPS.

Endotoxin tolerance causes a complex reprogramming of inflammatory responses (34). Proinflammatory cytokine expression is downregulated, whereas there is no change or even an increase in the expression of anti-inflammatory genes, antimicrobial genes, and genes mediating phagocytosis (34). Indeed, microarray analysis of tolerant versus nontolerant monocytes demonstrate a host of genes that are downregulated, whereas other genes are not downregulated (38). The net result of endotoxin tolerance appears to prevent host organ injury while maintaining antimicrobial functions. However, almost all the information regarding endotoxin tolerance is derived from gene expression in functionally mature monocytes or macrophages. Because endotoxin tolerance is an adaptive host response, the characteristics of endotoxin tolerance might vary depending on the inflammatory context. We previously reported that preterm sheep fetuses exposed to repeated doses of intraamniotic LPS demonstrate endotoxin tolerance and cross-tolerance to other Toll-like agonists (19, 20). The genes reported not to be downregulated after endotoxin tolerance include IL-10, IL-1ra, TGF-β1, serum amyloid, and others (38). Although IL-10 and IL-1ra were also downregulated in the chronic *U. parvum* + LPS animals in our study, TGF-β1 increased relative to controls. Another class of genes not downregulated during endotoxin tolerance is the TRIF-mediated IFN-inducible genes (39). Furthermore, IFN signaling negatively regulates TLR4 signaling and can abrogate endotoxin tolerance in blood monocytes (40, 41).

In the present experiment however, the pattern of expression of the IFN-inducible genes CXCL9 (monokine induced by IFN-γ) and CXCL10 (IFN-γ-inducible protein 10) mRNA expression in the lung after chronic *U. parvum* + LPS exposure was similar to the other proinflammatory cytokines. These results suggest that chronic exposure to *U. parvum* induced downregulation of LPS responses were more global in nature than previously reported in endotoxin tolerance.

Both intracellular negative regulators and extracellular soluble factors have been implicated in the mechanism of endotoxin tolerance. Extracellular/humoral factors that potentially mediate endotoxin signaling include steroid hormones (42), heat shock protein 70 (43), and IL-10 (44). The plasma cortisol levels (data not shown) and lung expression of IL-10 mRNA did not increase in these fetal lambs. However, TGF-β1 expression in the airways was higher in the lambs with downregulated LPS responses compared with controls. These data are consistent with the known anti-inflammatory properties of TGF-β1 (31). We previously reported increased expression of TGF-β1 and the downstream mediator phospho-SMAD2 after exposure to IA LPS (45). Both TGF-β1 and phospho-SMAD2 are detected in multiple lung cell types in the epithelium and the interstitium of fetal lung. The expression of mRNAs for the receptor TLR4 or the downstream transcription factors IRAK-1, IRAK-4, or IRAK-M in the lung did not change. Similarly, a growing list of intracellular mediators including MyD88s (46), IRAK-M (33), single Ig IL-1 receptor related (47), suppressor of cytokine signaling-1 (48), and others have been proposed as mediators of endotoxin tolerance. We previously reported that TLR4 mRNA is expressed ubiquitously in most of the lung cell types in the preterm fetus (49). In the absence of cell-type expression data for downstream mediators of TLR signaling, a caveat in the interpretation of changes in expression is that whole-lung homogenates used for quantification of mRNAs may dilute the changes in the relatively nonabundant recruited inflammatory cells.

Regulatory T cells expressing FOXP3 are abundant in the fetal circulation and downregulate inflammatory responses (50). However, in our study, acute or chronic exposure to *U. parvum* did not change FOXP3+ cells in the mediastinal lymph node or the lung (data not shown). Another mechanism for endotoxin tolerance is chromatin remodeling, changes in histone acetylation, and methylation induced gene silencing (38, 40). Although the precise molecular pathways of downregulated endotoxin responses in chorioamnionitis remain to be determined, increased TGF-β1 expression was demonstrated in the lungs of fetal lambs with downregulated LPS responses.

In contrast to sepsis, exposure of the fetus to bacterial components in chorioamnionitis is via the epithelia of the airway, the chorioamnion, and the gastrointestinal tract but not the vascular compartment. The resulting systemic inflammatory response is a mild increase in cytokines and acute phase reactants rather than the cytokine storm associated with sepsis in the adult (51, 52). Liver expression of acute-phase reactant genes CRP and serum amyloid A3 and plasma haptoglobin increased after IA LPS as expected, but the expression was not downregulated in lambs exposed to chronic *U. parvum* + LPS. These findings suggest that liver, the major source of acute-phase reactants, was not subject to downregulated LPS responses after exposure to chronic *U. parvum*. However, the blood monocytes from these chronic *U. parvum* + LPS animals were poorly responsive to LPS. Taken together, these findings suggest that LPS responsiveness in the fetus differed in different organs.

There are several clinical implications of our findings. Although *Ureaplasma* spp are the organisms most commonly associated with chorioamnionitis, ~60% of cases with chorioamnionitis have coinfection with ureaplasma and other microorganisms (3). Our findings suggest the possibility of decreased fetal inflammation in response to Gram-negative organisms with concomitant ureaplasma exposure. In contrast, analogous to adults with sepsis (36), diminished innate immune responses could cause adverse fetal outcomes. Similarly, postnatal nosocomial sepsis occurs in ~25% of very low birth weight preterm neonates (53). Our data suggest the possibility that ureaplasma chorioamnionitis could diminish innate immune responses and thereby increase the susceptibility of preterm infant to nosocomial sepsis. In summary, the experiments demonstrate a novel finding that a chronic exposure to intraamniotic *U. parvum* decreases both in vivo and in vitro lung endotoxin responsiveness in a preterm fetus.

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**Disclosures**

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**References**


