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# Intra-amniotic *Ureaplasma parvum*-Induced Maternal and Fetal Inflammation and Immune Responses in Rhesus Macaques

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**Background.** Although *Ureaplasma* species are the most common organisms associated with prematurity, their effects on the maternal and fetal immune system remain poorly characterized.

**Methods.** Rhesus macaque dams at approximately 80% gestation were injected intra-amniotically with 10<sup>7</sup> colony-forming units of *Ureaplasma parvum* or saline (control). Fetuses were delivered surgically 3 or 7 days later. We performed comprehensive assessments of inflammation and immune effects in multiple fetal and maternal tissues.

**Results.** Although *U. parvum* grew well in amniotic fluid, there was minimal chorioamnionitis. *U. parvum* colonized the fetal lung, but fetal systemic microbial invasion was limited. Fetal lung inflammation was mild, with elevations in CXCL8, tumor necrosis factor (TNF)  $\alpha$ , and CCL2 levels in alveolar washes at day 7. Inflammation was not detected in the fetal brain. Significantly, *U. parvum* decreased regulatory T cells (Tregs) and activated interferon  $\gamma$  production in these Tregs in the fetus. It was detected in uterine tissue by day 7 and induced mild inflammation and increased expression of connexin 43, a gap junction protein involved with labor.

**Conclusions.** *U. parvum* colonized the amniotic fluid and caused uterine inflammation, but without overt chorioamnionitis. It caused mild fetal lung inflammation but had a more profound effect on the fetal immune system, decreasing Tregs and polarizing them toward a T-helper 1 phenotype.

**Keywords.** chorioamnionitis; intrauterine infection; decidua; fetal immunology; regulatory T-cells.

Prematurity is the leading cause of infant mortality and morbidity globally [1]. Intrauterine infection or inflammation is associated with 40% of preterm labor [2]. Its most common cause is *Ureaplasma* species [3, 4]. Lower genital colonization with *Ureaplasma* is correlated with preterm labor [5, 6]. In addition to preterm labor, intrauterine infection or inflammation due to *Ureaplasma* species is also associated with an increased incidence of chronic lung disease [7] and gastrointestinal [8] and neurologic complications in preterm infants [9]. However, there is considerable variation in clinical manifestations from intrauterine *Ureaplasma* infection, eliciting minimal inflammatory responses without preterm labor in some women while causing robust inflammation in others [4].

Similar to effects in humans, intra-amniotic injection of live *Ureaplasma parvum* in pregnant sheep also caused chorioamnionitis of variable severity and fetal inflammation [10].

However, there was no clear microbial factor that could explain the variability in host response, because *U. parvum* strains isolated from the amniotic fluid of sheep with severe versus mild chorioamnionitis failed to retain their initial virulence on repeated injection in pregnant sheep [11]. One possibility for the variable response is a variable host immune response, but this has not been systematically studied.

The most relevant animal model for the study of placenta and fetal immune responses is the rhesus monkey (*Macaca mulatta*), owing to close similarities to humans in immunologic and reproductive biologic characteristics [12–14] and the availability of reagents to perform in-depth immune studies. In addition, intra-amniotic injection of *U. parvum* serovar 1 to chronically catheterized rhesus monkeys caused efficient amniotic colonization, preterm delivery, histologic chorioamnionitis, a systemic fetal inflammatory response, and fetal pneumonitis [15]. The pathologic effects described in monkeys were more severe than those in sheep. However, immune responses were not characterized in depth. Therefore, to better understand maternal and fetal inflammation and immune responses to *Ureaplasma* infection, we injected *U. parvum* serovar 1 into the amniotic fluid of pregnant rhesus macaques to characterize immune responses to acute (3-day) and subacute (7-day) exposures to intra-amniotic *U. parvum*.

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## METHODS

### Animals

Animal procedures were approved by the Institutional Animal Care and Use Committee at the University of California, Davis. Animals were not colonized with *Ureaplasma* before inoculation based on nasal, rectal, and vaginal screening by culture and polymerase chain reaction (PCR). By ultrasound guided intra-amniotic injection, time-mated female rhesus macaques (n = 18) were given either 1 mL of saline solution (controls) or *U. parvum* serovar 1 ( $1 \times 10^7$  colony-forming units per animal, diluted in 1 mL of saline solution; kindly provided by K. B. W.) (Table 1). Hysterotomies were performed 3 or 7 days after *U. parvum* injection at about 130 days gestational age (80% term gestation). None of the animals displayed any overt preterm labor or sickness. Dams and fetuses were euthanized, and tissues collected at necropsy. For fetal alveolar wash, the left main stem bronchus was cannulated, and the lung was inflated to near total lung capacity with normal saline solution and aspiration of the fluid. The alveolar wash was repeated 3 times, and the fluid was pooled.

### *Ureaplasma* Culture and PCR

Samples of amniotic fluid, chorioamnion, fetal lung, spleen, uterus, and brain were received in the University of Alabama at Birmingham Diagnostic Mycoplasma Laboratory on dry ice after being stored frozen at  $-80^\circ\text{C}$  in 10B broth. Quantitative cultures amniotic fluid and chorioamnion were performed using 10B broth and A8 agar [16]. Brown granular colonies grown on A8 agar after 1–3 days of incubation, typical of *Ureaplasma* species, were definitively identified as *U. parvum* by

real-time PCR, as described by Xiao et al [17]. Amniotic fluid, chorioamnion, fetal lung, brain, spleen, and uterus were also analyzed by real-time PCR for direct *U. parvum* detection [17], using the gene target *UU063*, which encodes a highly conserved hypothetical protein found in all 4 *U. parvum* serovars but not in *Ureaplasma urealyticum*.

### Cell Isolation and Culture

Fetal spleen and thymus were diced and dissociated with a pestle into cell suspensions, passed through 70- $\mu\text{m}$  cell strainers, and washed in culture medium (Roswell Park Memorial Institute 1640 medium) containing 10% fetal calf serum, 100 IU/mL penicillin, 100 IU/mL streptomycin, and 2 mmol/L glutamine. Blood mononuclear cells were isolated from approximately 10 mL of heparinized fetal blood by means of Ficoll-Hypaque (GE Healthcare) gradient centrifugation within 3 hours of collection. In spleen samples, red blood cell lysis was performed with ammonium chloride–potassium carbonate–ethylenediaminetetraacetic acid (Lonza BioWhittaker).

Cells were rested overnight at  $37^\circ\text{C}$  in air with 5% carbon dioxide. After overnight culture, viability was consistently  $>90\%$  (Trypan blue exclusion test). For cytokine measurements, cells were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) and 750 ng/mL ionomycin (Calbiochem) for 5 hours, with 10  $\mu\text{g}/\text{mL}$  brefeldin A (Sigma-Aldrich) and 1  $\mu\text{L}/\text{mL}$  monensin  $\times 1000$  (eBioscience) added for the last 4 hours.

### Flow Cytometry Analysis of Immune Cells

A cocktail of conjugated antibodies validated for the rhesus macaque was used (<http://www.nhpreagents.org/NHP/clonelist.aspx?ID=15>): anti-CD3 (SP34-2), anti-CD4 (L200), anti-CD45 (D058-1283), anti-Ki67 (B56) (BD Biosciences); anti-interferon (IFN)  $\gamma$  (B27), anti-forkhead box (FOX) P3 (PCH101), and anti-CD8 $\alpha$  (RPA-T8) (eBioscience). Cells were treated with human IgG to block Fc receptors and stained for surface markers. Cells were then washed and fixed with fixation/permeabilization buffer followed by permeabilization buffer (eBioscience) before staining for intracellular markers and analysis (FACS Fortessa2; BD Biosciences). Live cells were gated based on forward- and side-scatter properties and the absence of fluorescence using the LIVE/DEAD Fixable Dead Cell Stain kit (Invitrogen).

To evaluate T-cell subsets in the decidua, cells were cultured overnight at  $37^\circ\text{C}$  and 5% carbon dioxide in Dulbecco's modified Eagle medium/F12 medium containing 10% fetal bovine serum, 100 IU/mL penicillin, 100 IU/mL streptomycin, and 2 mmol/L glutamine, because the dissociation/protease digestion can down-regulate CD4/CD8 receptors [18–20]. Data analysis was performed using FACSDiva (version 6.1.2; BD Biosciences) and FlowJo (version 9.9.4; Tree Star) software.

### Immunohistochemistry

Immunohistochemistry was performed as described elsewhere [21]. Briefly, tissue sections were incubated with anti–connexin

**Table 1. *Ureaplasma parvum* Infection and Inflammation**

Variable	Control (n = 8)	<i>U. parvum</i> Infected <sup>a</sup>	
		3 d (n = 5)	7 d (n = 5)
Fetal weight, mean (SD) g	331 (7.2)	348 (44)	341 (18)
Gestational age at delivery, mean (SD), d	132 (0.8)	132 (4.3)	131 (1.2)
Histologic chorioamnionitis, No.	1/8	0/5	1/5
<i>U. parvum</i> quantitative culture, median (IQR), CFUs $\times 10^3/\text{mL}$			
Fetal membrane	0	0 (0–0.2)	0.2 (0–0.9)
Amniotic fluid	0	0.1 (0.03–84)	3900 (1875–9275)
<i>U. parvum</i> detection by PCR, No.			
Fetal membrane	0/8	4/5	4/5
Amniotic fluid <sup>b</sup>	0/5	5/5	5/5
Maternal uterus	ND	0/5	3/5
Fetal lung	ND	4/5	5/5
Fetal spleen	ND	0/5	2/5
Fetal thalamus	ND	1/5	0/5

Abbreviations: CFUs, colony-forming units; IQR, interquartile range; ND, not determined; PCR, polymerase chain reaction; SD, standard deviation; *U. parvum*, *Ureaplasma parvum*.

<sup>a</sup> *U. parvum* ( $1 \times 10^7$  CFUs) was given via intra-amniotic injection.

<sup>b</sup> Data from only 5 of 8 control animals.

**Table 2. Amniotic Fluid Cytokine Levels**

Study Group <sup>a</sup>	Cytokine Level, Median (IQR), ng/mL						
	IL-1 $\beta$	IL-6	CXCL8	TNF- $\alpha$	CCL2	IL-1ra	IL-10
Control (n = 5)	0.4 (0.01–0.4)	396 (301–893)	138 (67–946)	0.1 (0.1–21)	954 (521–1152)	208 (142–289)	0.1 (0.1–3)
<i>U. parvum</i> infected, 3 d (n = 5)	0.4 (0.01–0.4)	360 (210–859)	147 (119–700)	0.1 (0.1–6.4)	673 (512–878)	90 (27–213)	0.1 (0.1–0.1)
<i>U. parvum</i> infected, 7 d (n = 5)	0.6 (0.6–1.2)	532 (426–1006)	635 (323–1486)	2.5 (5.8–55.9)	2231 (1416–2541) <sup>b</sup>	257 (172–644)	0.1 (0.1–27.6)

Abbreviations: IL-1 $\beta$ , interleukin 1 $\beta$ ; IL-1ra, interleukin 1ra; IL-6, interleukin 6; IL-10, interleukin 10; IQR, interquartile range; TNF, tumor necrosis factor; *U. parvum*, *Ureaplasma parvum*.

<sup>a</sup> *U. parvum* ( $1 \times 10^7$  colony-forming units) was given via intra-amniotic injection.

<sup>b</sup>  $P < .05$  vs control.

43 (Abcam Ab11370, 1:750), anti-myeloperoxidase (MPO; Cell Marque 289A-75; 1:250), anti-CD3 (Dako Cytomation, A0452; 1:100), or anti-CD68 antibody (Dako Cytomation, M0814; 1:1000) in 4% normal goat serum at 4°C overnight, followed by incubation with anti-rabbit IgG secondary antibody diluted 1:200 in 2% serum for 2 hours at room temperature. After further washing, antigen-antibody complexes were visualized using a Vectastain ABC peroxidase kit (Vector Laboratories). Antigen detection was enhanced with nickel-diaminobenzidine, followed by incubation with Tris-cobalt. Slides were counterstained with Nuclear Fast Red for photomicroscopy. Blinded digital images of the uteri (5 nonoverlapping random fields per animal and 5 animals per group) were quantified for inflammatory cells, namely neutrophil (MPO<sup>+</sup>), monocyte/macrophage (CD68<sup>+</sup>), and T cells (CD3<sup>+</sup>). Connexin 43 staining intensity was also quantified based on color threshold and expressed as stained area relative to total area (Metamorph; Molecular Devices).

#### Histologic Evaluation of Fetal Membranes for Chorioamnionitis

Hematoxylin-eosin–stained sections of fetal membranes were scored in a blinded manner (by S. G. K.) for chorioamnionitis using criteria outlined by Redline et al [22], based on numbers and depth of neutrophil infiltration of the tissue.

#### RNA Isolation, Complementary DNA Generation, and Quantitative Reverse-Transcription PCR

Total RNA was extracted from snap frozen fetal lung, maternal uterus, and chorioamnion-decidua after homogenizing in TRIzol. RNA concentration and quality was measured with a Nanodrop spectrophotometer. Reverse-transcription (RT) of RNA was performed using Verso complementary DNA synthesis kits. Quantitative RT-PCR used a StepOnePlus Real Time PCR system following standard cycling conditions. Quantitative RT-PCR was performed with rhesus-specific TaqMan gene expression primers (Thermo-Fisher Scientific). The Eukaryotic 18S ribosomal RNA was the endogenous control for normalization of the target RNAs, and a sample from a control animal (administered intra-amniotic saline solution) was used as a calibrator.

#### Cytokine Measurements

Cytokine/chemokine concentrations in the amniotic fluid and alveolar wash fluid were determined using Luminex technology with Non-Human Primate Multiplex kits (Millipore). Concentrations were calculated from standard curves for recombinant proteins.

#### Statistical Analyses

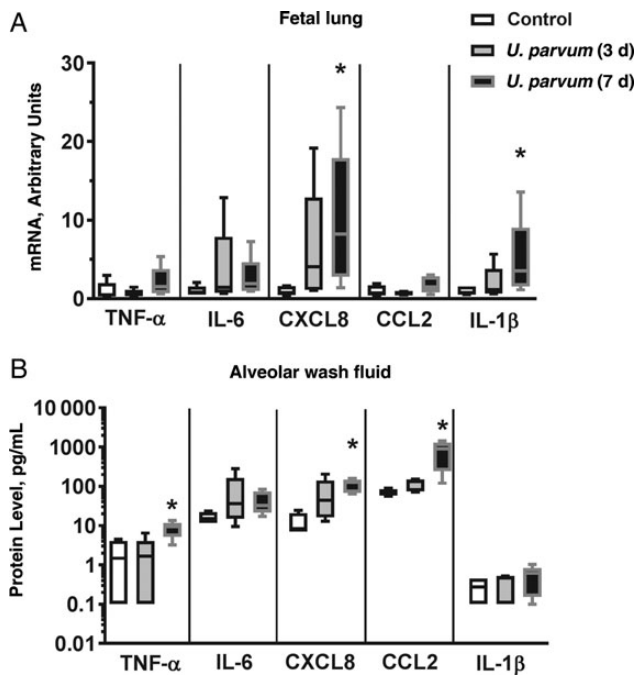
GraphPad Prism software (version 7.01) was used to graph and analyze statistical significance. Values were expressed as mean (standard deviation) or median (interquartile range). Statistical differences between groups were analyzed using nonparametric Mann–Whitney *U* tests. Results were considered significantly different at  $P \leq .05$ . Considering the limited number of animals per group, we also report trends ( $0.05 < P < .10$ ).

## RESULTS

#### Intra-amniotic Injection of *U. parvum* Caused Minimal Inflammation in the Amniotic Cavity

The amniotic fluids from all the *U. parvum*-injected animals were *U. parvum* positive by PCR, whereas *U. parvum* was not detected in the amniotic fluid or fetal membranes from any of the control animals. The amniotic fluid *U. parvum* titer increased by 4 logs from 3 to 7 days after intra-amniotic injection, with wide variation for both the 3- and 7-day *U. parvum* groups (Table 1). In marked contrast to its robust growth in the amniotic fluid, *U. parvum* grew poorly from the fetal membranes. Although the PCR was positive in fetal membranes from 4 of 5 animals in each group, *U. parvum* could be cultured from only 1 of 5 fetal membranes from the 3-day group, and 2 of 5 from the 7-day group.

Histologic chorioamnionitis was present in only 1 of 5 animals in the 7-day *U. parvum* group (Table 1). Compared with controls, CCL2 (monocyte chemoattractant protein [MCP]) was the only chemokine increased in the amniotic fluid of the 7-day *U. parvum* group, although all measured cytokines and chemokines were numerically higher at 7 days (Table 2). Neutrophils were not detected in the amniotic fluid of either the control or *U. parvum*-infected animals (both 3- and 7-day groups). The messenger RNA (mRNA) expression of several



**Figure 1.** Intra-amniotic *Ureaplasma parvum*-induced modest fetal lung inflammation. *A*, Messenger RNAs (mRNAs) were isolated from the right lower lobe of the fetal lung. Quantitative polymerase chain reaction was performed using rhesus-specific TaqMan probes. The values in box-and-whisker plots were first internally normalized to the endogenous 18S RNA, and the resultant values for the experimental animals were shown as fold increase compared with the mean value for the controls. *B*, Box-and-whisker plots show cytokine protein levels in alveolar wash fluid from controls and *U. parvum*-infected animals at 3 and 7 days. Boxes represent 25th–75th percentiles; horizontal lines, medians; and the whiskers, represent 5th and 95th percentile values ( $n=5$  per group); \* $P<.05$  (Mann–Whitney  $U$  test). A strong trend toward significance was observed for IL-6 protein levels at 7 days, CXCL8 protein levels at 3 days, and CCL2 protein levels at 3 days in alveolar wash fluid (all  $P=.06$  vs controls; Mann–Whitney  $U$  test). Abbreviations: IL-1 $\beta$ , interleukin 1 $\beta$ ; IL-6, interleukin 6; TNF, tumor necrosis factor.

cytokines and chemokines (interleukin 1 $\beta$  [IL-1 $\beta$ ], interleukin 6, CXCL8, TNF- $\alpha$ , and CCL2) were not increased in the fetal membranes (chorion-amnion decidua) at either 3 or 7 days of intra-amniotic *U. parvum* infection (Supplementary Table 1). Moreover, there were no changes in the frequency of macrophages, neutrophils, natural killer (NK) cells, NKT cells, T cells, B cells, or regulatory T cells (Tregs) in the dissociated chorio-decidua cells either 3 or 7 days after intra-amniotic *U. parvum* (Supplementary Table 2), further confirming the histologic scoring for chorioamnionitis.

#### Intra-amniotic *U. parvum* Infection Induced Modest Fetal Lung Inflammation

Consistent with aspiration of the infected amniotic fluid, *U. parvum* was detected in the fetal lung of all *U. parvum*-inoculated animals except 1 at 3 days. CXCL8 (interleukin 8) (8-fold) and IL-1 $\beta$  (4-fold) mRNAs increased at 7 days after inoculation with no change at 3 days, compared with control animals (Figure 1A). Similarly, alveolar wash protein levels for TNF- $\alpha$  (6-fold),

CXCL8 (interleukin 8) (15-fold), and CCL2 (MCP-1) (14-fold) increased from control levels 7 days after intra-amniotic inoculation with *U. parvum* (Figure 1B). Unlike the mRNA expression, there were no significant changes in IL-1 $\beta$  protein in the alveolar wash fluid. Bronchoalveolar lavage fluid did not contain neutrophils or macrophages in either the controls or *U. parvum*-inoculated animals at 3 days. Only 1 in 5 fetuses in the *U. parvum* 7-day group had a few neutrophils and monocytes in the bronchoalveolar lavage fluid. There were no changes in mRNA expression for surfactant proteins A, B, C, and D (data not shown).

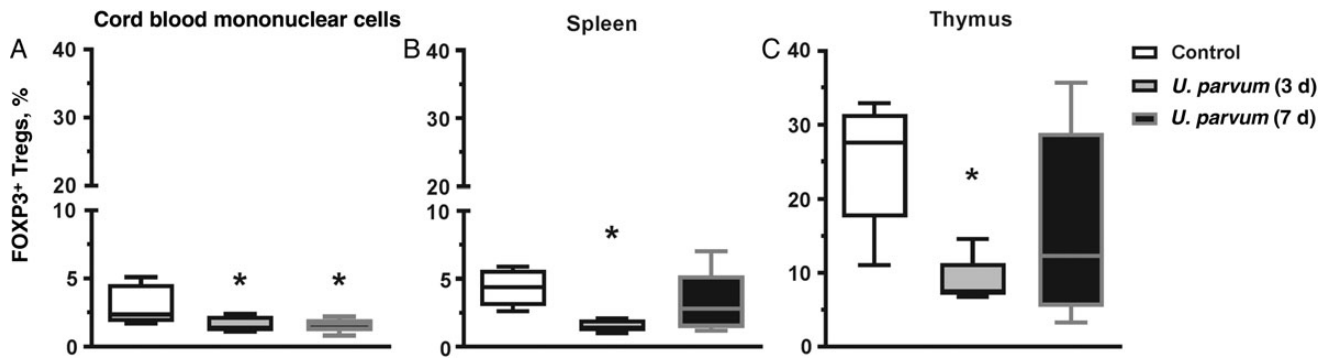
#### Intra-amniotic *U. parvum* Infection Did Not Induce Fetal Brain Inflammation

*U. parvum* was detected with PCR in only 1 of 5 fetal brains (thalamus) 3 days after exposure and none of 5 7 days after exposure. Brain inflammation was measured by quantitative RT-PCR for IL-1 $\beta$ , CCL2, TNF- $\alpha$ , interleukin 10, cyclooxygenase 2, and prostaglandin E synthase 2 in the cortex, periventricular white matter, thalamus, hippocampus, and cerebellum. Exposure to intra-amniotic *U. parvum* did not increase the mRNA expression of proinflammatory cytokines in any of these brain areas (data not shown). Similarly, none of the cytokines increased in the cerebrospinal fluid (data not shown). Immunohistology demonstrated that there was no infiltration of CD3<sup>+</sup> or CD4<sup>+</sup> lymphocytes, nor any cellular changes in microglia (Iba-1), oligodendrocytes (Olig2), astrocytes (GFAP), neurons (NeuN), myelin basic protein, or apoptosis (caspase 3) in any of the brain areas (data not shown) (methods for brain analysis provided in Supplementary information).

#### Intra-amniotic *U. parvum* Infection Decreased Tregs Proportion in Thymus and Peripheral Lymphoid Organs, and Activated a Th1-Type Profile in the Fetal Tregs

Systemic dissemination of the intra-amniotic *U. parvum* in the fetus was evident in only 2 of 5 fetal spleens at 7 day and none at 3 days. We previously reported that exposure to chorioamnionitis (from IL-1 $\beta$  or lipopolysaccharide [LPS]) rapidly decreased Treg proportions in fetal lymphoid organs in preterm rhesus monkey [23, 24]. We thus analyzed the effect of *U. parvum* exposure on immune cells and subsets. Compared with control animals, *U. parvum* exposure did not change the absolute number of fetal thymocytes or the proportions of the major thymocyte subsets (ie, progenitors, double-negative, double-positive, or single-positive CD4<sup>+</sup> or CD8<sup>+</sup> thymocytes; data not shown). However, the proportion of FOXP3<sup>+</sup> thymic Tregs within the single-positive CD4<sup>+</sup> thymocytes was significantly decreased 3 days after *U. parvum* infection, with a variable nonsignificant decrease at 7 days (Figure 2C).

In the periphery (cord blood, spleen), *U. parvum* exposure had no effect on total CD8 and CD4 T-cell frequency and absolute counts, or on NK cells (CD3<sup>-</sup>CD8<sup>-</sup>NKG2A<sup>+</sup>). Similarly, compared with controls, the frequency of monocytes (CD14<sup>+</sup>),

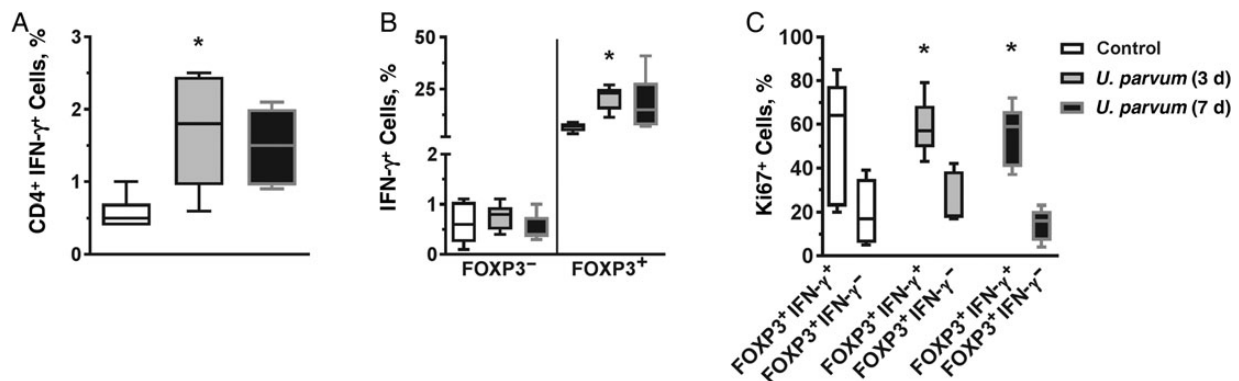


**Figure 2.** Intra-amniotic *Ureaplasma parvum* infection decreased fetal regulatory T cell (Treg) proportion. Cell suspensions from the thymus, spleen and, blood mononuclear cells were analyzed by flow cytometry. Box-and-whisker plots show frequency of Tregs (defined as forkhead box [FOX] P3<sup>+</sup>) within total CD4<sup>+</sup>CD3<sup>+</sup> T cells in cord blood mononuclear cells (A), spleen (B), and total CD4 single-positive thymocytes (C), defined as CD34<sup>-</sup>CD7<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup>CD8a<sup>-</sup> cells. Boxes represent 25th–75th percentiles; horizontal line, medians; and whiskers, 5th and 95th percentile values (n = 5–6 per group). \*P < .05 vs controls (Mann–Whitney U test).

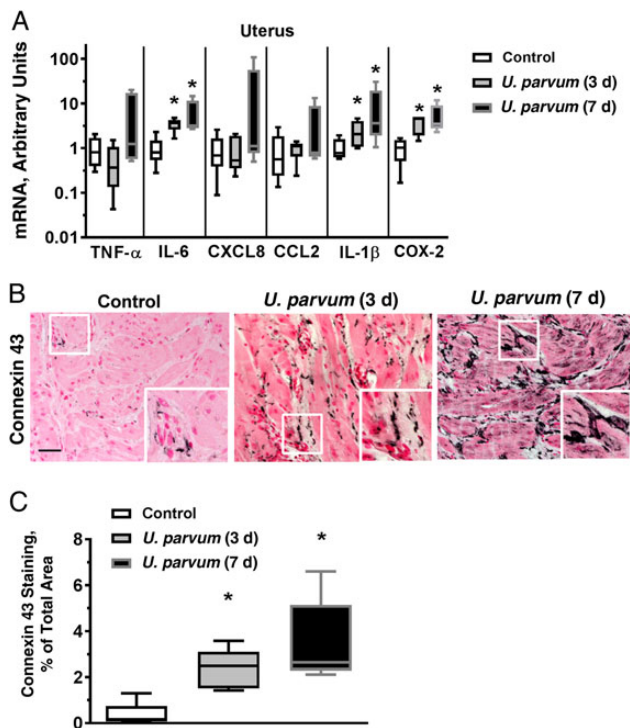
plasmacytoid dendritic cells (HLA-DR<sup>+</sup>CD11c<sup>-</sup>CD123<sup>+</sup>), and myeloid dendritic cells (HLA-DR<sup>+</sup>CD11c<sup>+</sup>CD123<sup>-</sup>), as well as their activation status (CD40, CD80, CD86 expression), was unchanged in the blood or lymphoid organs of *U. parvum*-exposed fetuses (data not shown). However, as in the thymus, the *U. parvum*-exposed animals at 3 days also had significantly lower proportion of CD4<sup>+</sup>FOXP3<sup>+</sup> blood and spleen Tregs (Figure 2A and 2B; gating strategy in Supplementary Figure 1A). Absolute numbers of splenic Tregs in *U. parvum* animals were also significantly decreased compared to control animals (×10<sup>6</sup>/g) (5.2 [2.2] vs 2.5 [1.9]; P = .03, mean [SD]). Similar decreases in Tregs were also detected in the mesenteric lymph nodes 3 days after intra-amniotic *U. parvum* infection (data not shown). Again, as in the thymus, peripheral Tregs started to rebound 7 days after inoculation (Figure 2).

We next investigated whether *U. parvum* changed the expression profile of cytokines (IFN-γ and interleukin 17 [IL-17], 2,

and 22) in fetal T and NK cells after a short PMA/ionomycin ex vivo challenge. The percentage of IFN-γ-expressing CD4<sup>+</sup> splenic T cells was significantly increased at day 3 (Figure 3A; gating strategies in Supplementary Figure 1B), and similar increases were observed in the blood and mesenteric lymph nodes (data not shown). This activation was selective for IFN-γ, because the frequency of cells expressing interleukin 2, IL-17, and interleukin 22 did not change (data not shown). In the *U. parvum* 7-day group, the proportion of IFN-γ<sup>+</sup>CD4<sup>+</sup> T cells tended to remain high, although the difference from controls was not significant (Figure 3A). We previously showed that fetal Tregs express effector cytokines, and the percentage of these multifunctional Tregs increases with inflammation [24]. Therefore, we analyzed which subsets contribute to the enhanced IFN-γ expression. Within the CD4<sup>+</sup> population, up-regulation of IFN-γ expression was restricted to the FOXP3<sup>+</sup> cells (Figure 3B). Similarly, expression of IFN-γ by CD8<sup>+</sup> T or NK



**Figure 3.** Intra-amniotic *Ureaplasma parvum* activated a T-helper (Th) 1-type profile in fetal CD4<sup>+</sup> forkhead box (FOX) P3<sup>+</sup> regulatory T cells (Tregs). Cell suspensions were prepared from spleen and stimulated with PMA and ionomycin for 5 hours, with brefeldin A and monensin added for the last 4 hours. Expression of intracellular interferon (IFN) γ was analyzed in different subsets by flow cytometry. A, Frequency of total IFN-γ<sup>+</sup> cells within CD4<sup>+</sup>CD3<sup>+</sup> T cells. B, Frequency of IFN-γ<sup>+</sup> expressing cells within the FOXP3<sup>+</sup>CD4<sup>+</sup>CD3<sup>+</sup> and FOXP3<sup>-</sup>CD4<sup>+</sup>CD3<sup>+</sup> T-cell populations. C, Frequency of the cell cycle protein Ki67<sup>+</sup> cells within IFN-γ<sup>+</sup> or IFN-γ<sup>-</sup> FOXP3<sup>+</sup> expressing splenic Tregs. Boxes represent 25th–75th percentiles; horizontal lines, medians; and whiskers, 5th and 95th percentile values (n = 5–6 per group); \*P < .05 vs controls. In control animals, IFN-γ<sup>+</sup>FOXP3<sup>+</sup> Tregs also tended to express more Ki67 than their IFN-γ<sup>-</sup> counterparts (P = .09; Mann–Whitney U test).



**Figure 4.** Intra-amniotic *Ureaplasma parvum*-induced modest inflammation and increased connexin 43 in the uterus. **A**, Messenger RNAs (mRNAs) were isolated from full-thickness uterine tissue including the adherent decidua. Quantitative PCR was performed using rhesus-specific Taqman probes. The values were first internally normalized to the endogenous 18S RNA, and the resultant values for the experimental animals were shown as fold increase compared with the mean control value. \* $P < .05$  vs controls (Mann-Whitney  $U$  test). **B**, Immunostaining was performed for the contraction-associated gap junction protein connexin 43. Representative photomicrograph is shown for each study group; the inset in each panel shows a higher-magnification photomicrograph. Note more intensely stained areas of connexin 43 in both *U. parvum* groups compared with control (scale bar, 50  $\mu$ m). **C**, Image quantitation for the connexin 43 immunostaining was performed and expressed as stained area relative to total area (mean of 5 random fields per animal was used as representative value for each animal). Boxes represents 25th–75th percentile; horizontal lines, medians; and whiskers, 5th and 95th percentile values ( $n = 5$  per group). \* $P < .05$  vs controls (Mann-Whitney  $U$  test). Abbreviations: IL-1 $\beta$ , interleukin 1 $\beta$ ; IL-6, interleukin 6; TNF, tumor necrosis factor.

cells was unchanged (data not shown). These IFN- $\gamma$ <sup>+</sup>FOXP3<sup>+</sup> cells had increased cell cycling compared with their IFN- $\gamma$ <sup>-</sup> counterparts, as indicated by their enhanced expression of Ki67 (Figure 3C; gating strategy in Supplementary Figure 1C).

#### Intra-amniotic Injection of *U. parvum* Caused Uterine Inflammation

Although *U. parvum* was not detected with PCR in any of the maternal uteri at 3 days, it was present in 3 of 5 uteri at 7 days. Interestingly, in contrast to the very limited effects on fetal membranes, intra-amniotic *U. parvum* increased IL-1 $\beta$  (3–4 fold), interleukin 6 (4–5 fold), and cyclooxygenase 2 (4–5 fold) mRNA expression in the uterus at both 3 and 7 days after exposure, compared with control animals (Figure 4A). Uterine TNF- $\alpha$ , MCP-1, and CXCL8 mRNA expression was also variably but not significantly increased. Similarly, the

mRNA expression for prostaglandin F receptor (PTGFR), inducible nitric oxide synthase, matrix metalloproteinase 2, 8, and 9, and vascular endothelial growth factor A and C in the uterus did not change (Supplementary Table 3). There were modest but significant increases in neutrophil (MPO<sup>+</sup>), monocyte/macrophage (CD68<sup>+</sup>), and T cells (CD3<sup>+</sup>) in uterine tissue (Supplementary Figure 2). The inflammatory cells seemed to be located within the capillaries interspersed between smooth muscle cells rather than infiltrating the tissue. Connexin 43, a gap junction protein, is essential for parturition [25]. Compared with controls, intra-amniotic *U. parvum* increased the expression of connexin 43 at both 3 and 7 days, as detected by immunohistochemistry (Figure 4B and 4C). However, no change was seen in the expression of other genes associated with uterine contraction, namely, oxytocin receptor, myosin light chain kinase, RhoA, Rho-associated coiled-coil containing protein kinase 1 and 2, and finger E-Box binding homeobox 1 and 2 (Supplementary Table 3).

#### DISCUSSION

Although *Ureaplasma* species are the most common organisms associated with intrauterine infection [3, 4], their contribution to perinatal pathology is still not well understood [26, 27]. Part of the problem is that in humans, naturally occurring *Ureaplasma* infections are commonly polymicrobial [3, 28]. We used a preterm rhesus macaque model and a well characterized *U. parvum* serovar to explore these relationships, and our results provide a nuanced picture of immune changes and inflammation in different fetal-maternal compartments with intrauterine infection with *U. parvum*.

The lack of chorioamnionitis was surprising given prior reports by Novy et al [15, 29] demonstrating severe chorioamnionitis in rhesus macaques at about the same gestational age. We used the same strain, dose, and source of *U. parvum* and the organism efficiently grew in the amniotic fluid, but unlike those studied by Novy et al, the rhesus macaques in our study were not instrumented. The lack of infiltration of inflammatory cells in the decidua after *U. parvum* is in sharp contrast to a robust deciduitis induced by intra-amniotic IL-1 $\beta$  [21]. Our results in the rhesus are thus more reminiscent of modest inflammatory response in the maternal compartments in fetal sheep [10, 30] and consistent with a finding that  $\geq 25\%$  of amniocenteses or placentas with *Ureaplasma* species in humans did not have histologic chorioamnionitis or amniotic inflammation [4, 31]. We also found no significant growth of *U. parvum* in the rhesus fetal membranes. These observations raise a question regarding the ability of *U. parvum* to directly attach to and signal intact amniotic epithelium in vivo, although *U. parvum* was reported to signal dissociated amniotic epithelial cells in vitro [32]. The presence of other microorganisms along with *U. parvum* in the amniotic fluid or placenta may modulate its virulence [4, 28].

Consistent with fetal airways being in contact with contents of the amniotic fluid, we demonstrated fetal lung colonization with *U. parvum* in all the animals. However, the fetal inflammation was modest, similar to our data in the fetal sheep [30]. Surfactant protein mRNAs were also not increased after intra-amniotic *U. parvum* infection. *U. parvum*-induced lung maturational changes were measured after chronic exposure (45 days) to intra-amniotic *U. parvum* in sheep, but not after shorter exposures (3–14 days) [30, 33]. Similarly, although dissemination of *U. parvum* in the fetal central nervous system was described clinically [9], we could not detect invasion of *U. parvum* in the fetal brain, nor could we detect inflammation or injury responses in the brain. In contrast, intra-amniotic LPS causes brain inflammation, microglia activation, and impaired myelination in the fetal rhesus macaque [34], and fetal sheep [35]. Novy et al also infrequently detected *U. parvum* in fetal brains of rhesus macaques, with culture or PCR [15].

An important finding of our study is that *U. parvum* decreased the CD4<sup>+</sup>FOXP3<sup>+</sup> Treg population, in both the thymus and the periphery. Wolfs et al [36] reported that the proportion of CD4<sup>+</sup>FOXP3<sup>+</sup> cells decreased in the gut of *U. parvum*-exposed fetal sheep at 3 and 7 days after infection, but this study did not characterize fetal immune responses in depth. In our current study, the effect on Tregs was surprising because the levels of inflammatory cytokines measured in the amniotic fluid and fetal plasma were unchanged or modestly increased after *U. parvum* infection, although we might have missed an early increase (before 3 days) in amniotic fluid cytokines, as previously reported [15]. Furthermore, *U. parvum*-induced Treg changes (mainly at 3 days) preceded higher amniotic fluid colonization rates (colony-forming unit counts were higher on day 7 than on day 3). Similarly to *U. parvum* infection, both intra-amniotic IL-1 $\beta$  and LPS diminished the proportion and absolute number of fetal Tregs [23, 24]. However, *U. parvum* induced a IFN- $\gamma$  response in Tregs (with approximately 25% of fetal spleen Tregs adopting a bifunctional phenotype), whereas intra-amniotic LPS induced a predominant IL-17 response [24].

This finding is new and provocative, because neonatal T-helper (Th) 1 responses have long been considered impaired, mostly because neonatal antigen-presenting-cells seem deficient at producing the major pro-Th1 cytokine, interleukin 12 (reviewed in [37]). However, this concept has been challenged recently, because IFN- $\gamma$  expression was detected in a population of activated neonatal T cells in the absence [38] or presence [39] of exposure to maternal inflammation. These results thus suggest the existence of a subset of cells that can develop a Th1 phenotype early in life, and this response may be increased in the presence of inflammation. Future studies will be needed to better understand the mechanisms that dictate the response of fetal T cells to in utero inflammation, particularly the emergence and functional characteristics of these “inflammatory” Tregs.

The effects of intra-amniotic *U. parvum* infection on the uterus is a novel observation. There were small but significant increases in proinflammatory cytokine mRNA expression, increases in leukocytes, mostly in the capillaries, and increased expression of connexin 43 in the uterus after exposure to *U. parvum*. These findings are consistent with intrauterine *U. parvum* inducing preterm labor in rhesus monkey [15, 29]. However, we did not detect overt preterm labor in any of the intra-amniotic *U. parvum* groups. Because uterine inflammation was detected in the absence of *U. parvum* demonstrable by PCR at 3 days, the results suggest that indirect signaling to the uterus via the amniotic compartment may play a role in pathogenesis.

In conclusion, *U. parvum* efficiently colonized rhesus macaque amniotic fluid, causing maternal uterine inflammation but no chorioamnionitis. Despite the modest fetal inflammation, Treg populations decreased in the fetal thymus and peripheral lymphoid organs, adopting an “inflammatory” phenotype. These results mirror the moderate pathogenicity of *Ureaplasma* in human pregnancy. However, our results also suggest that infections with indolent microorganisms such as *U. parvum* can affect the developing fetal immune system. Their influence might be underestimated owing to the absence of overt chorioamnionitis at birth.

### Supplementary Data

Supplementary materials are available at <http://jid.oxfordjournals.org>. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

### Notes

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