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Intra-Amniotic IL-1β Induces Fetal Inflammation in Rhesus Monkeys and Alters the Regulatory T Cell/IL-17 Balance

Suhas G. Kallapur,* Pietro Presicce,* Paranthaman Sentharamaraikannan,* Manuel Alvarez,* Alice F. Tarantal,†,‡ Lisa M. Miller,*§ Alan H. Jobe,* and Claire A. Chougnet*

Very low birth weight preterm newborns are susceptible to the development of debilitating inflammatory diseases, many of which are associated with chorioamnionitis. To define the effects of chorioamnionitis on the fetal immune system, IL-1β was administered intra-amniotically at ~80% gestation in rhesus monkeys. IL-1β caused histological chorioamnionitis, as well as lung inflammation (infiltration of neutrophils or monocytes in the fetal airways). There were large increases in multiple proinflammatory cytokine mRNAs in the lungs at 24 h postadministration, which remained elevated relative to controls at 72 h. Intra-amniotic IL-1β also induced the sustained expression of the surfactant proteins in the lungs. Importantly, IL-1β significantly altered the balance between inflammatory and regulatory T cells. Twenty-four hours after IL-1β injection, the frequency of CD3+CD4+FOXP3+ T cells was decreased in lymphoid organs. In contrast, IL-17A–producing cells (CD3+CD4+, CD3+CD4−, and CD3−CD4− subsets) were increased in lymphoid organs. The frequency of IFN-γ–expressing cells did not change. In this model of a single exposure to an inflammatory trigger, CD3+CD4+FOXP3+ cells rebounded quickly, and their frequency was increased at 72 h compared with controls. IL-17 expression was also transient. Interestingly, the T cell profile alteration was confined to the lymphoid organs and not to circulating fetal T cells. Together, these results suggest that the chorioamnionitis-induced IL-1/IL-17 axis is involved in the severe inflammation that can develop in preterm newborns. Boosting regulatory T cells and/or controlling IL-17 may provide a means to ameliorate these abnormalities.

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Novy and colleagues (30–33) showed that intra-amniotic injection of IL-1β to the fetal macaques induced chorioamnionitis and preterm labor. However, these studies did not explore fetal tissues in detail or immune responses. Therefore, we used an intra-amniotic exposure to IL-1β in fetal macaques to define the effects of chorioamnionitis on the fetal immune system.

Materials and Methods

Animals and sample collection

All animal procedures conformed to the requirements of the Animal Welfare Act, and protocols were approved prior to implementation by the Institutional Animal Care and Use Committee at the University of California, Davis. Normally cycling, adult female rhesus monkeys (Macaca mulatta; n = 17) with a history of prior pregnancy were bred and identified as pregnant, using established methods (28). Activities related to animal care were performed as per standard California National Primate Research Center operating procedures. Animals were randomized to receive either 10 μg human rIL-1β (PeproTech, Rocky Hill, NJ) in 1 ml sterile saline or 1 ml sterile saline by ultrasound-guided intra-amniotic injection at 130 ± 2 d gestational age, which is ~80% of term gestation (term: 165 ± 10 d). The dose of 10 μg was chosen based on previous work done in macaques (30, 33). Furthermore, chorioamnionitis and fetal inflammation are induced by 100 μg IL-1β in fetal sheep, which are ~10 times larger than fetal macaques (16). Of note, high levels of IL-1β have been measured in amniotic fluid (34), but the relative exposure of the fetal compartment to IL-1β in humans remains unclear.

Hysterotomies were performed, and the gestational sac was removed aseptically 24 or 72 h postadministration of IL-1β (Table I). Fetuses were euthanized with an overdose of pentobarbital, and peripheral blood and tissues (spleen, mesenteric and mediastinal lymph nodes [LN], lung lobes, amniotic fluid) were collected. Bronchoalveolar lavage fluid (BALF) was prepared following tissue collection. Each LN was dissected, and cells were mechanically detached from the surrounding membrane using a scalpel and fine tweezers. Spleen was diced and dissociated into a homogenous cell suspension using a pestle. Cell suspensions were passed a scalpel and fine tweezers. Spleen was diced and dissociated into a homogenous cell suspension using a pestle. Cell suspensions were passed through 70-μm cell strainers and washed in culture media (RPMI 1640) containing 10% FCS, 100 IU/ml penicillin, 100 IU/ml streptomycin, and 2 mmol/l glutamine. Using ~10 ml heparinized fetal blood, PBMCs were isolated using Ficoll-Hypaque (GE Healthcare–Piscataway, NJ) gradient centrifugation within 3 h of collection. RBC lysis was performed as needed, using ammonium chloride/potassium carbonate/EDTA (Lanza BioWhittaker, Pittsburgh, PA). Cells were rested overnight at 37˚C and 5% CO2. After overnight culture, viability was consistently >85% (trypan blue exclusion test). For cytokine measurements, cells were stimulated with 50 ng/ml PMA (Sigma-Aldrich, St. Louis, MO) and 750 ng/ml ionomycin (Calbiochem, San Diego, CA) for 5 h, with 10 μg/ml brefeldin A (Sigma-Aldrich) and 1 μg/ml monensin 1000× (eBioscience, San Diego, CA) added for the last 4 h.

Flow cytometry

The following Ab clones were used: SP34-2 (anti-CD3), L200 (anti-CD4), M-A251 (anti-CD25), PCH101 (anti–FOXP3), B56 (Ki67), BN3 (anti–CTLA-4), B27 (anti–IFN-γ), and 6C4CAP17 (anti–IL-17A). Cells were treated with 20 μg/ml human IgG to block FcR, stained for surface markers for 30 min at 4˚C in PBS, washed, and fixed in 1% paraformaldehyde. Intracellular staining for FOXP3 was performed using the eBioscience reagents, according to the manufacturer’s instructions. A negative isotype control for FOXP3 (CD3+ CD4− cells) was used as a reference to establish the cut-off for FOXP3+ cells in CD3+CD4+ T cells, as previously described (35, 36). Intracellular staining for cytokines (IL-17, IFN-γ) was performed using Cytofix/Cytoperm (BD Bioscience), according to the manufacturer’s instructions. All Abs were titrated for optimal detection of positive populations and mean fluorescent intensity. At least 250,000 events were recorded for each sample. Doubles were excluded on the basis of scatter properties, and dead cells were excluded using LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen). Data were analyzed using FlowJo software (TreeStar, Ashland, OR).

Immunohistochemistry

Immunostaining protocols used lung paraffin sections (5 μm) from 10% formalin-fixed tissues that were deparaffinized and rehydrated using a series of xylene and alcohol washes. Ag Spireal was performed using citrate buffer (pH 6) or 1 mM EDTA buffer (pH 8). Tissues were pretreated with 3% H2O2 to inactivate endogenous peroxidases. The sections were incubated with anti-human CD3 (1:50; DakoCytomation, Glostrup, Denmark), CD4 (1:50; Leica Biosystems, Nussloch, Germany), CD56 (1:100; AbD Serotec, Kidlington, U.K.), CD68 (1:250; DakoCytomation), or myeloperoxidase (MPO; 1:200; Cell Marque, Rockland, CA) in 10% normal goat serum at 4˚C overnight, followed by biotin-labeled secondary Ab. Immunostaining was visualized using a VECTASTAIN ABC Peroxidase Elite kit (Vector Laboratories) to amplify the Ag–Ab complexes. The Ag staining was further enhanced with nickel-diaminobenzidine, followed by Tris-cobalt, and the nuclei were counterstained with Nuclear Fast Red. In addition, H&E staining was performed for lung tissue and chorioamnion. The severity of chorioamnionitis was graded using the criteria of Redline et al. (37).

RNA isolation, cDNA generation, and quantitative RT-PCR

Total RNA was isolated from the fetal right lung using TRIzol reagent (Invitrogen, Carlsbad, CA). The quality and yield of the RNA were assessed with a NanoDrop fluorospectrophotometer (NanoDrop, Wilmington, DE). One microgram of total RNA was used to synthesize cDNA using a Verso cDNA kit (Thermo Scientific), following the manufacturer’s protocol. Quantitative RT-PCR was carried out with the TaqMan Gene Expression Master Mix and Rhesus Specific TaqMan Gene Expression Assay, performed in a 7300 Real Time PCR System (all from Applied Biosystems, Carlsbad, CA). Cycling conditions consisted of a 2-min incubation at 50°C, followed by a 10-min incubation at 95°C and 40 cycles of alternating temperatures of 95°C for 15 s and 60°C for 1 min. Eukaryotic 18S rRNA (Applied Biosystems) was used as an endogenous control for normalization of the target RNAs, and a sample from the control group was used as a calibrator.

Cytokine and cortisol measurement

Cytokine/chemokine concentrations were determined by Lumines (Millipore, Billerica, MA), according to the manufacturer’s protocol. Concentrations were calculated from standard curves using recombinant proteins and are expressed in pg/ml. Cortisol was measured after extraction of plasma with ethyl ether using ELISA (Oxford Biomedical Research, Rochester Hills, MI), as per the manufacturer’s instructions. This test operates on the basis of competition between the enzyme conjugate and the cortisol in plasma for a limited number of binding sites on the Ab-coated plate.

Statistical analyses

Parametric or nonparametric tests were used depending on the normality of data distribution. ANOVA or Kruskal–Wallis tests were used first to determine whether the three groups were significantly different, followed by one-to-one group comparisons using t tests or Mann–Whitney tests.

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Table I. Description of the animals included in the study

<table>
<thead>
<tr>
<th>Animals</th>
<th>Maternal Parity (No.)</th>
<th>Maternal Age (y)</th>
<th>Maternal Weight (kg)</th>
<th>Gestational Age (d)</th>
<th>Fetal Weight (g)</th>
<th>Fetus Gender (male/female)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n = 7)</td>
<td>4 (3–9)</td>
<td>9 (6–16)</td>
<td>7.4 (5.3–11.3)</td>
<td>131 (121–132)</td>
<td>317 (285–356)</td>
<td>5/2</td>
</tr>
<tr>
<td>IL-1 at 24 h (n = 6)</td>
<td>3 (1–12)</td>
<td>8 (7–16)</td>
<td>7.8 (5.4–9.3)</td>
<td>130 (128–132)</td>
<td>324 (263–405)</td>
<td>3/3</td>
</tr>
<tr>
<td>IL-1 at 72 h (n = 4)</td>
<td>4 (1–6)</td>
<td>9 (6–11)</td>
<td>8.4 (5.5–10.8)</td>
<td>129 (128–130)</td>
<td>319 (293–443)</td>
<td>1/3</td>
</tr>
</tbody>
</table>

Data are median (range). Maternal weights, ages, and parity were recorded at the time when the animals were included in the study.

The p values were determined using the Kruskal–Wallis test.

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2 INTRA-AMNIOTIC IL-1β EFFECTS ON FETAL IMMUNE RESPONSES

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Results were considered significantly different for \( p \) values < 0.05. However, due to the limited number of samples per group, trends are reported (\( p \) between 0.05 and 0.15).

Results

**Intra-amniotic IL-1β induces chorioamnionitis**

The intra-amniotic injection of IL-1β or saline was performed in multiparous macaques of similar weight and age (Table I). Mean fetal weights at tissue collection were not different between groups. Confirming previous results with this model (33), IL-1β altered ratios of Treg and IL-17–expressing cells in lymphoid organs.

**Intra-amniotic IL-1β induces lung inflammation and maturation**

Control fetuses had no neutrophils or monocytes within the fetal airways. In contrast, intra-amniotic IL-1β increased neutrophils and monocytes in the BALF at 24 h, and inflammatory cells remained high at 72 h (Fig. 2A). IL-1β also induced indicators of lung maturation, as indicated by the sustained increased expression of surfactant protein (A, B, C, and D) mRNAs in the fetal lungs (Fig. 3). We measured plasma cortisol levels because glucocorticoids are given routinely to induce lung maturation in the preterm fetus (1). Control fetuses had low plasma cortisol levels (mean ± SE: 3.7 ± 0.2 µg/dl). Upon exposure to intra-amniotic IL-1β, the plasma cortisol increased significantly to 5.8 ± 0.3 µg/dl at 24 h (\( p < 0.01 \), versus controls), with a decrease to 4.7 ± 0.9 µg/dl at 72 h.

Intra-amniotic IL-1β also induced large increases in multiple proinflammatory cytokine mRNAs in the fetal lungs (Fig. 2B–G). Indeed, the expression of IL-1β, IL-6, IL-8, and TNF-α mRNAs was increased by >50-fold at 24 h compared with controls. MCP-1 was also increased significantly, but to a lesser degree. These proinflammatory cytokines remained significantly higher than control values at 72 h, although they were decreased compared with the 24-h time point. The mRNA results were confirmed by measuring cytokine levels in the BALF (Fig. 4A). The pattern of increased proinflammatory cytokine proteins reflected the increased mRNA levels at 24 h, with levels decreasing at 72 h. IL-10 and IL-1RA also increased modestly, with the increase in IL-10 apparent at 72 h. In contrast, no significant increases were detected in IL-17, IL-4, IL-10, IFN-γ, or IL-2 mRNA expression in the lungs (data not shown).

Double immunostaining for MPO and CD68 was used to characterize the lung-infiltrating cells (Fig. 5, Supplemental Fig. 1). Consistent with the BALF data, the lungs of control fetuses contained very few neutrophils (expressing just MPO) or macrophages (CD68⁺ or CD68⁺MPO⁺ cells). Upon exposure to intra-amniotic IL-1β, the neutrophilic (MPO⁺CD68⁻) population increased by >6-fold at 24 h and >4-fold at 72 h, and these cells were ~70–80% myeloid cells in the IL-1β–exposed animals. Activated macrophages (CD68⁺MPO⁺) were also significantly more abundant in the IL-1β–exposed animals. These data suggest infiltration of fetal lung, predominantly by neutrophils and some activated macrophages, upon exposure to IL-1β. T cells were not detected in the lung or the BALF of control animals, and their numbers did not increase after IL-1β exposure (data not shown).

**Intra-amniotic IL-1β alters ratios of Treg and IL-17–expressing cells in lymphoid organs**

Intra-amniotic IL-1β did not change the overall proportion of CD4⁺CD3⁺ T cells in the mediastinal LN, mesenteric LN, spleen, or PBMCs at either time point (data not shown; all \( p > 0.20 \), ANOVA). However, IL-1β altered the frequency of Tregs (defined as the percentage of FOXP3⁺ cells in the CD3⁺CD4⁺ population) in the mediastinal LN, mesenteric LN, and spleen. Treg frequency at 24 h decreased by ~2-fold compared with controls (\( p \) values ranging from 0.06 to 0.09, Mann–Whitney test, Fig. 6). In this model of single exposure to an inflammatory trigger, Treg fre-
frequency was increased at 72 h compared with 24 h, reaching levels generally higher than those in controls (Fig. 6).

In control fetuses, as expected, CD3+CD4+FOXp3+ cells expressed high levels of CD25 and low levels of CD127 (Fig. 7A, 7B). They also expressed high levels of CD39 and CTLA-4 compared with their FOXp3− counterparts (Fig. 7A, 7B). Intra-amniotic IL-1β did not change the expression of these markers in Tregs or non-Tregs (data not shown). Interestingly, in control animals, Tregs tended to express higher levels of the cell-cycling marker Ki67 than did non-Tregs (Fig. 7A, 7B); IL-1β significantly increased the levels of Ki67 in Tregs, but not non-Tregs, at 24 h (Fig. 7C).

In contrast, 24 h after IL-1β injection, the percentages of IL-17A+ cells in the CD3+CD4+ and CD3−CD4− cells purified from the mediastinal LN, mesenteric LN, and spleen were increased compared with those in controls (Fig. 8, Supplemental Fig. 2 for representative staining). Again, as described above for Tregs, IL-17 induction was transient, because its expression at 72 h was similar to that seen in control animals (Fig. 8B). Baseline, unstimulated IL-17 expression was similar between groups in all organs (data not shown; all p > 0.15, Kruskal–Wallis test). Interestingly, IL-1β did not significantly affect IFN-γ-expressing cells in any of the lymphoid tissues studied (Table II).

Immune changes induced by IL-1β in tissues and lungs are not detected in fetal blood

Because peripheral blood is the only compartment easily accessible in premature human newborns, we determined whether the changes occurring in tissues were also detectable in the blood. In contrast to the lymphoid organs, intra-amniotic IL-1β did not change Treg frequency in PBMCs (Fig. 6D), nor did it induce IL-17 expression in PBMCs (Fig. 8D). Similarly, levels of proinflammatory cytokines modestly increased in the blood compared with the levels measured in the BALF. Plasma IL-6 was increased significantly at 24 h in IL-1–treated animals (Fig. 4B), but the other proinflammatory cytokines that were increased in the fetal BALF of treated animals (IL-1β, IL-8, TNF-α, GM-CSF, MCP-1) did not increase simultaneously in fetal plasma (data not shown). Interestingly, plasma levels of IL-1RA and IL-10 increased modestly (Fig. 4B), following a similar pattern as the BALF (Fig. 4A).

**Discussion**

Until recently, the chorioamnionitis-associated infection/inflammation that initiates the events leading to preterm labor was thought to be an ascending colonization of the endometrium, leading to a diffuse invasion of the chorion and amnion prior to dissemination to the amniotic fluid and the fetus (38). However, more recent pathological evaluations support a different model of focal colonization (infection) of the chorioamnion, with dissemination into the amniotic fluid (and to the fetus) prior to the development of diffuse chorioamnionitis (39, 40). This distinction in the pathways to infection/inflammation of the fetal compartment is important because it changes the direction of proinflammatory signal for preterm labor from the uterus to the fetal compartment. Therefore, a simple model of intra-amniotic exposure may best reflect the focal membrane contamination that progresses to disseminated amniotic fluid infection, followed by diffuse chorioamnion colonization prior to preterm labor (41, 42). Using such a model, acute chorioamnionitis caused fetal inflammatory responses and profound changes in T cell responses at 24 h, which reverted to normal by 72 h. Intra-amniotic IL-1β induced a sustained response in fetal tissues, because chorioamnionitis was more pronounced at 72 h than at 24 h, and lung cytokine expression remained increased at 72 h.

To our knowledge, our study provides the first experimental evidence that increased IL-17 responses could be induced in the fetus following chorioamnionitis. IL-1β and IL-6 are potent inducers of IL-17 (43, 44), and both cytokines were upregulated in the chorioamnion of IL-1-exposed macaques (data not shown).
We also found significant upregulation of these cytokines at 24 h in the lungs, with lower expression at 72 h. Because of the short half-lives of cytokines, it is likely that the endogenously produced proinflammatory cytokines were responsible for the IL-17 induction. In contrast to its effect on IL-17, intra-amniotic IL-1β did not change IFN-γ expression by fetal cells. These data fit with a recent report indicating that CD4+ T cells from extremely preterm infants have a low Th1 bias, but a significant Th17 bias, which was linked to their high baseline levels of RORγt and STAT3 mRNA (45). Interestingly, in our model, several cell populations expressed IL-17, including CD3+CD4+, CD3+CD4−, and CD3−CD4− subsets. We did not further characterize these CD3−IL-17+ cells, and further analysis will be warranted to address this question. They may be NK cells or fetal lymphoid tissue inducers, which both lack CD3 and are known IL-17 producers (46). Alternatively, they may be T cells that internalized the TCR upon activation. Macrophages also produce IL-17 in some cases, but these IL-17+ macrophages are found in inflamed tissues or tumors and not in lymphoid organs (47–49). Neutrophils also produce IL-17 (50), but the CD3−IL-17+ cells in the lymphoid organs did not have the distinct forward/scatter characteristics of neutrophils.

Taken together, our results suggest that the preterm nonhuman primate fetus has the capacity to mount an IL-17 response, although it was transient in this model using a single sterile cytokine as the inflammatory trigger. This experimental exposure would not be expected to induce IL-23, the most potent cytokine to stabilize the Th17 phenotype, because IL-23 production is mainly prompted by TLR-mediated stimulation of APCs (51). Neonatal mononuclear cells are high producers of IL-23, while being poor IL-12 producers (52–54). Of note, our recent results in a sheep model showed relatively long-lasting effects of LPS-induced inflammation on the fetal thymus, because upregulation of IL-17 mRNA and downregulation of FOXP3 mRNA persisted several days after the exposure (55). However, we acknowledge that such an acute model of inflammation does not fully reproduce human chorioamnionitis, which is often a chronic fetal exposure to inflammatory challenges. In the future, it will be important to use the rhesus macaque model to explore the fetal immune system in the setting of placental colonization by Mycoplasma or Ureaplasma, which are frequently associated with preterm birth and poor outcomes (56).

Compared with controls, the frequency of CD4+FOXP3+ cells in all lymphoid organs decreased 24 h after IL-1 injection, while rebounding at 72 h. This pattern was reciprocal to the frequency of Th17 cells in the same lymphoid organs. These CD4+FOXP3+ cells are likely Tregs, because they expressed significantly higher levels of Treg functional markers compared with their FOXP3− counterparts (Fig. 5), although suppression assays could not be performed due to the lack of available fetal Treg populations.

### Figure 6
Intra-amniotic IL-1β transiently decreases Treg frequency in lymphatic organs. Data are mean (SE) Treg frequency (defined as percentage of FOXP3+ cells in the CD3+CD4+ population) for each organ (n = 4–6/group). Significant p values or trends are shown (Mann–Whitney test).

### Figure 7
Phenotypic characterization of Treg and non-Tregs. (A) Significant example of Treg gating. Live cells were first identified by the absence of LIVE/DEAD stain and forward scatter expression. Then CD3+ CD4+ T cells were gated, and FOXP3+ cells were identified in this gate. Cut-off for FOXP3+ cells in CD3+CD4+ T cells was set up according to a negative biological population, as previously described (35, 36). Expression of CD25, CD127, CD39, CTLA-4, and Ki67 was measured in gated Tregs (CD3+CD4+FOXP3+) or non-Tregs (CD3+CD4−FOXP3−). (B) Mean (SE) percentage of Treg and non-Tregs expressing CD25, CD127, CD39, CTLA-4, and Ki67 in spleens of control animals. Significant p values or trends are shown (Mann–Whitney tests). (C) Effect of IL-1β on Ki67 expression of Treg and non-Tregs. Data are mean (SE) percentage of Tregs or non-Tregs expressing Ki67 (n = 4–6/group). Significant p values or trends are shown (Mann–Whitney test).

### Figure 8
Intra-amniotic IL-1β transiently increases the frequency of IL-17+ cells in lymphatic organs. Percentages of IL-17A+ cells were measured by flow cytometry in gated CD3+CD4+, CD3+CD4−, and CD3−CD4− subsets after short in vitro polyclonal restimulation of cells purified from the mediastinal LN (A), mesenteric LN (B), spleen (C), and PBMCs (D). Data are mean (SE) percentage of IL-17+ cells in each subset (n = 4–6/group). Significant p values or trends are shown (Mann–Whitney test). ND, Not done.
performed to confirm these findings because of limited cell yields. Thus, these results in a nonhuman primate model confirm our previous findings in fetal sheep, in which we showed that chorioamnionitis decreases Treg frequency in thymus and gut (16, 21, 22). Notably, a recent clinical study showed a significantly reduced proportion of Tregs in the intestinal mucosa of premature infants with necrotizing enterocolitis compared with age-matched controls (57). Importantly, Treg proportions correlated with increased mucosal proinflammatory cytokine levels, and they were normalized in the healing phase, at the time of reanastomosis. Taken together, these results support the hypothesis that the strong inflammatory environment in the fetus inhibits Treg development and homeostasis and that decreased Treg proportions may contribute to inflammatory disorders in premature babies. Mechanisms underlying the changed Treg/Th17 balance have not been elucidated, but they could involve the key metabolic sensor and regulator hypoxia-inducible factor 1\(a\), which plays a key role in T cell fate determination (58, 59). Regulation of this pathway could include direct signaling of Tregs by IL-1, because it can regulate levels of hypoxia-inducible factor 1\(a\) (60), and activated Tregs express high levels of IL-1R (61, 62). Interestingly, we found that Tregs tended to express higher baseline levels of KI67 than did CD4\(^+\)FOXP3\(^+\) cells, a result in agreement with recent studies showing that Tregs express high levels of cell cycle molecules in vivo in both mice and humans (63–65). KI67 expression in Tregs, but not in non-Tregs, was further increased 24 h after IL-1 injection compared with controls. This selective increase in cell cycle activation could explain the rapid rebound of Tregs at 72 h. Further experiments will be necessary to clarify the signaling pathways triggered by chorioamnionitis that are able to affect T cell phenotypes in the fetus.

Notably, T cell changes in lymphoid tissues were not detected in fetal blood. This result is consistent with recent reports that the composition of T cell subsets is different in the lymphoid organs and the circulating blood of human fetuses, as evidenced by the fact that the frequency of memory CD4\(^+\) T cells detectable in their lymphoid tissues (spleen, LNs, GALTs) is higher than that found in the fetal blood (66, 67). Similarly, several proinflammatory cytokines were greatly increased in the BALF but not in the fetal blood, with the exception of plasma IL-6, which reached levels indicative of fetal inflammatory response syndrome in the human (>11 pg/ml) (8, 68). Studies of immune modulation following in utero activation will not be easy to conduct in preterm humans, because the blood is the only immune compartment relatively accessible in this fragile population.

Another observation from this study was the increase in the mRNA for the four surfactant-associated proteins following IL-1\(\beta\)-induced chorioamnionitis. There are two independent inducers of fetal lung maturation that are important clinically: maternal corticosteroid treatments and intrauterine exposure to inflammation (69). Bry et al. (70) first identified inflammation as an inducer of lung maturation following intra-amniotic injection of IL-1\(\beta\) in fetal rabbits. Lung maturation was detected as increased mRNA for surfactant proteins and improved pressure-volume curves. Subsequently, intra-amniotic inflammation was demonstrated to consistently cause lung maturation in rodents and sheep (15, 71). To our knowledge, this is the first demonstration of lung maturation induced experimentally in a nonhuman primate. The magnitude of the increase in mRNA for the surfactant proteins is comparable to that reported in other species (72). Based on our experience with fetal sheep (15), we did not measure pressure-volume curves or surfactant lipids because they would not be expected to increase within 72 h. Although cortisol modestly increased after intra-amniotic IL-1\(\beta\), it is unlikely that it mediated the lung maturation because plasma levels were ~10-fold lower than those reported in normal-term humans (73).

Together, our data incriminate the chorioamnionitis-induced IL-1/IL-17 axis in the inflammation developing in very preterm newborns. The capacity of fetuses to mount a robust IL-17 response might constitute a double-edged sword because it would afford them protection against infections by bacteria and fungus, but it could also play a role in the development of the devastating inflammatory disorders that are often seen in these preterm babies. Our results also suggest that boosting Tregs and/or controlling IL-17 may help to control these pathologies. Furthermore, we demonstrate that the fetal rhesus macaque is a suitable model to study mechanisms of inflammation in human fetuses.

Acknowledgments
We thank the staff at the California National Primate Research Center for invaluable contributions, particularly Sarah Davis, for help with all aspects of animal management. We also thank all members of our respective laboratories for logistical help during the study.

Disclosures
The authors have no financial conflicts of interest.

Table II. IFN-γ-producing cells in mediastinal LN, mesenteric LN, and spleen

<table>
<thead>
<tr>
<th>Tissues</th>
<th>CD3(^{+})CD4(^{-}) (%)</th>
<th>CD3(^{+})CD4(^{-}) (%)</th>
<th>CD3(^{+})CD4(^{-}) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mediastinal LN</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>0.7 (0.4–1.9)</td>
<td>1.3 (0.2–4.0)</td>
<td>1.7 (0.4–2.5)</td>
</tr>
<tr>
<td>IL-1β at 24 h</td>
<td>0.9 (0.2–1.4)</td>
<td>1.1 (0.4–3.4)</td>
<td>3.7 (0.9–7.5)</td>
</tr>
<tr>
<td>p Value</td>
<td>0.58</td>
<td>0.74</td>
<td>0.27</td>
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<tr>
<td><strong>Mesenteric LN</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>0.6 (0.1–1.4)</td>
<td>4.24 (0.2–9.9)</td>
<td>3.5 (2.2–7.1)</td>
</tr>
<tr>
<td>IL-1β at 24 h</td>
<td>0.6 (0.4–0.9)</td>
<td>1.41 (0.4–8.4)</td>
<td>1.7 (1.2–3.7)</td>
</tr>
<tr>
<td>IL-1β at 72 h</td>
<td>0.3 (0.1–0.7)</td>
<td>0.53 (0.3–0.8)</td>
<td>4.2 (2.0–5.8)</td>
</tr>
<tr>
<td>p Value</td>
<td>0.30</td>
<td>0.23</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td></td>
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</tr>
<tr>
<td>Controls</td>
<td>1.8 (0.5–3.1)</td>
<td>4.0 (1.0–15.0)</td>
<td>4.6 (4.0–5.8)</td>
</tr>
<tr>
<td>IL-1β at 24 h</td>
<td>1.2 (0.5–6.2)</td>
<td>4.4 (0.8–5.1)</td>
<td>4.1 (1.2–12.4)</td>
</tr>
<tr>
<td>IL-1β at 72 h</td>
<td>0.6 (0.2–2.7)</td>
<td>1.4 (0.7–3.8)</td>
<td>6.1 (4.5–10.8)</td>
</tr>
<tr>
<td>p Value</td>
<td>0.45</td>
<td>0.38</td>
<td>0.46</td>
</tr>
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</table>
References


Suppl. Fig. 1: Representative photomicrographs of CD68 and myeloperoxidase (MPO) staining in fetal lung sections. Lung sections from paraffin embedded blocks were stained for CD68 (green) MPO (red) and DAPI (blue) to visualize nuclei. Representative staining from the following groups of animals are shown: saline control (A-C), IL-1β 24h (D-F), and IL-1β 48h (G-I). Very few CD68 or MPO positive cells were detected in the control lung sections and no double positive cells are seen in the merged frames. In contrast, IL-1β increased the MPO+ cells (red) and CD68+MPO+ cells (orange-yellow) in the lung. The MPO+ or CD68+ or CD68+MPO+ cells are shown with an arrow in the inset representing a magnification of the area shown in the larger frame. Please note the numerous red blood cells that are autofluorescent in the green channel (shown with asterisks). Magnification scale bar in A is 50µm.
Suppl. Fig. 2: Representative flow cytometry analysis of IL-17 and IFN-γ expression. Percentages of IL-17A+ and IFN-γ+ cells were measured by flow cytometry in gated CD3+CD4+, CD3+CD4− and CD3−CD4− subsets after short in vitro polyclonal restimulation of cells purified from the mediastinal LN of saline controls, IL-1 24h or IL-1 72h animals.