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 Sentharamaikannan, Manuel Alvarez, Alice F. Tarantal, Lisa M. Miller, Alan H. Jobe and Claire A. Chougnet

*J Immunol* published online 21 June 2013
http://www.jimmunol.org/content/early/2013/06/21/jimmunol.1300270

---

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2013/06/21/jimmunol.1300270.DC1

**Subscription**
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
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+γ, and CD3+CD4+γ 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. The Journal of Immunology, 2013, 191: 000–000.

Very preterm newborns frequently develop severe inflammatory diseases affecting multiple organs, including bronchopulmonary dysplasia, necrotizing enterocolitis, and postnatal sepsis (1). The connection between fetal inflammation and other morbidities of the premature infant, such as retinopathy of prematurity and cerebral palsy, is also of concern (2, 3). Although the origins of these pathologies are likely multifactorial, they are frequently associated with chorioamnionitis (4). Fetal inflammation has been assessed in clinical studies by measuring cytokine concentrations in amniotic fluid, neonatal plasma, and gastric and tracheal aspirates (5–7). Elevated levels of cytokines, such as IL-6, IL-8, and TNF-α, have been associated with chorioamnionitis (5, 8–12). Intra-amniotic injection of live organisms in the macaque induced IL-1 and caused preterm labor (13, 14). We showed in fetal sheep that chorioamnionitis induced with the intra-amniotic injection of LPS or IL-1 resulted in inflammation, particularly of the fetal lung, gut, skin, and chorioamnion (15–17). IL-1 was central to this inflammation, because blockade of IL-1 signaling in the amniotic compartment with a recombinant IL-1R antagonist (IL-1RA) largely inhibited the fetal lung and systemic inflammation caused by intra-amniotic LPS (18).

IL-1β has profound effects on the immune system, inducing chemokine and IL-6 production, which are particularly sensitive to IL-1β (reviewed in Ref. 19). Importantly, IL-1 appears essential to the generation of the Th17 response, given that T cells from mice deficient in IL-1R1 fail to express IL-17 upon Ag challenge (20). Therefore, we hypothesized that infection in utero would induce an inflammatory cascade that can cause preterm labor and activate the fetal immune system. A relevant observation in the fetal sheep chorioamnionitis model was a decrease in the frequency of regulatory T cells (Tregs) in the gut and thymus (16, 21, 22). However, detailed studies are impractical in the sheep because of the lack of reagents to interrogate the immune system. The rhesus macaque model offers an attractive alternative to evaluate immune modulation by chorioamnionitis because of the availability of many cross-reacting Abs and the high degree of similarity in the ontogeny of the immune system in rhesus macaques and humans. Indeed, by the second trimester of gestation, the lymphoid tissues of the rhesus monkey fetus have a complete repertoire of appropriately organized APCs, T cells, and B cells (23), similar to human fetuses (24). In contrast, development of lymphoid tissues is delayed in rodents (25). TLR and inflammasome systems are also conserved between nonhuman primates and humans (26, 27). Furthermore, many aspects of reproductive biology are very similar when comparing the rhesus macaque and humans (28, 29).
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 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 [LNs], lung lobes, placental bed, and amniotic fluid) were collected. Bronchoalveolar lavage fluid (BALF) was recovered by inflating the left lung to total lung capacity with normal saline, followed by fluid withdrawal. The procedure was repeated three times, and the fluids were pooled and used for cell counts and protein analysis.

Cell isolation and culture

Single-cell suspensions from spleen and mesenteric and mediastinal LNs were 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 p-10 needle. 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 mM/l glutamine. Using ~10 µl 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 (Lonzia 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 human IL-1β (PeproTech, Rocky Hill, NJ) and preincubated with 20 µg/ml brefeldin A (Sigma–Aldrich) and 1 µM monensin 1000x (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 (Ki-67), BNI3 (anti–CD25), L200 (anti-CD4), and preterm labor. They were performed with cells cultured using a p-10 needle. Cells were plated in 24-well plates at 1x 10⁶ cells/well and incubated for 1 h to allow for cell attachment. After washing, cells were fixed in 1% paraformaldehyde. Intracellular staining for FOXP3 was performed using the eBioscience reagents, according to the manufacturer’s instructions. A negative biological population 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 paraffinized and rehydrated using a series of xylene and alcohol washes. An overnight incubation of eBioscience reagents, according to the manufacturer’s instructions. A negative biological population 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).

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 using 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 Luminesix using Non-Human Primate Multiplex kits (Millipore, Billerica, MA), according to the manufacturer’s protocol. Concentrations were calculated from standard curves using recombinant proteins and were 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.

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.
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-
in PBMCs (Fig. 8D). Similarly, levels of proinflammatory cytokines in PBMCs (Fig. 6D), nor did it induce IL-17 expression in the lymphoid organs, 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+FOXP3+ cells expressed high levels of CD25 and low levels of CD127 (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).

FIGURE 3. Intra-amniotic IL-1β induces lung maturation. Target RNAs were measured by quantitative RT-PCR and normalized on the expression of eukaryotic 18S rRNA. A sample from the control group was used as a calibrator to calculate the fold changes. Data are mean (SE) fold change in RNA expression in the lung of each animal (n = 4–6/group). Significant p values or trends are shown (Mann–Whitney test).

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).

FIGURE 4. Median cytokine levels in fetal BALF and plasma. Mean (SE) cytokine levels (pg/ml) in the BALF (A) and plasma (B). For statistical analyses, undetectable values were assigned an arbitrary value of 1 pg/ml. *Significant difference between controls and IL-1–treated animals (24 or 72 h, Mann–Whitney test; n = 4–6/group). †Significant difference between 24- and 72-h time points (Mann–Whitney test).

FIGURE 5. Intra-amniotic IL-1β induces inflammatory cell influx in the lung. Lung sections were double immunostained with anti-MPO and anti-CD68. Data are mean (SE) cell counts/high-power field (HPF) of single- and double-positive populations. Intra-amniotic IL-1β increased MPO+ cells and MPO−CD68+ cells in the fetal lung (n = 4/group). Significant p values or trends are shown (Mann–Whitney test).
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), 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 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 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⁺ 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-17⁺ 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 or 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 1α, 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 1α (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-1β–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-1β 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-1β, 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>
</tr>
<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>p Value</td>
<td>0.30</td>
<td>0.23</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td></td>
<td></td>
<td></td>
</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>p Value</td>
<td>0.45</td>
<td>0.38</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Cells purified from the mediastinal LN, mesenteric LN, and spleen of controls or animals treated by intra-amniotic IL-1β for 24 or 72 h were stimulated in vitro with PMA and ionomycin. Percentages of IFN-γ⁺ cells were measured by flow cytometry in gated CD3⁺CD4⁺, CD3⁺CD4⁻, and CD3⁻CD4⁺ subsets. Data are median (range) percentage of IFN-γ⁺ cells in each subset (n = 4–6/group).

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


