Systemic interleukin-2 administration improves lung function and modulates chorioamnionitis-induced pulmonary inflammation in the ovine fetus

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Willems MG, Ophelders DR, Nikiforou M, Jellema RK, Butz A, Delhaas T, Kramer BW, Wolfs TG. Systemic interleukin-2 administration improves lung function and modulates chorioamnionitis-induced pulmonary inflammation in the ovine fetus. Am J Physiol Lung Cell Mol Physiol 310: L1–L7, 2016. First published October 30, 2015; doi:10.1152/ajplung.00289.2015.—Chorioamnionitis, an inflammatory reaction of the fetal membranes to microbes, is an important cause of preterm birth and associated with inflammation-driven lung injury. However, inflammation in utero overcomes immaturity of the premature lung by inducing surfactant lipids and lung gas volume. Previously, we found that lipopolysaccharide (LPS)-induced chorioamnionitis resulted in pulmonary inflammation with increased effector T cells and decreased regulatory T cell (Treg) numbers. Because Tregs are crucial for immune regulation, we assessed the effects of interleukin (IL)-2-driven selective Treg expansion on the fetal lung in an ovine chorioamnionitis model. Instrumented fetuses received systemic prophylactic IL-2 treatment [118 days gestational age (dGA)] with or without subsequent exposure to intra-amniotic LPS (122 dGA). Following delivery at 129 dGA (term 147 dGA), pulmonary and systemic inflammation, morphological changes, lung gas volume, and phospholipid concentration were assessed. IL-2 pretreatment increased the FoxP3+/CD3+ ratio, which was associated with reduced CD3+positive cells in the fetal lungs of LPS-exposed animals. Prophylactic IL-2 treatment did not prevent pulmonary accumulation of myeloperoxidase- and PU.1-positive cells or elevation of bronchoalveolar lavage fluid IL-8 and systemic IL-6 concentrations in LPS-exposed animals. Unexpectedly, IL-2 treatment improved fetal lung function of control lambs as indicated by increased disaturated phospholipids and improved lung gas volume. In conclusion, systemic IL-2 treatment in utero preferentially expanded Tregs and improved lung function of control lambs as indicated by increased disaturated phospholipids. These beneficial effects on lung function were maintained despite the moderate immunomodulatory effects of prophylactic IL-2 in the course of chorioamnionitis.

chorioamnionitis; regulatory T cells; interleukin-2; pulmonary inflammation; lung function
**Sampling protocol.** Fetal blood samples were taken at 24 h or 3, 5, or 7 days (end of experiment) after intra-amniotic LPS or saline administration using heparin-containing blood-collecting tubes. Plasma was prepared by centrifugation at 4,000 revolutions/min. Body weight of the fetus was recorded upon delivery. The thoracic cavity was opened, and a static pulmonary pressure-volume curve was performed. Lung gas volumes were recorded for every pressure and were corrected for body weight. Lungs were weighed, and the left lung was subjected to bronchoalveolar lavage. The right upper lobe (RUL) of the lungs was inflation fixated using 10% buffered formalin and paraffin embedded. Right lower lobe (RLL) tissue was embedded in optimal cutting temperature compound (OCT).

**Immunohistochemistry.** Paraffin-embedded RUL tissues (4 μm) were stained for CD3 (T cells), FoxP3 (Tregs), myeloperoxidase (MPO; activated neutrophils and monocytes), and PU.1 (maturation marker for myeloid cells). Protocols and information about antibodies are published previously (23, 30).

OCT-embedded RLL tissues (5 μm) were stained for CD25 [IL-2 receptor-α (IL-2Rα) (36)]. Slides were fixated in cold acetone, and endogenous peroxidase activity was blocked by 0.3% H2O2 in methanol. The rest of the protocol was the same as described previously for FoxP3 (30), with the exception that anti-CD25 (MCA2218; AbD Serotec, Kidlington, UK) was used.

CD3-, MPO-, and PU.1-positive cells were counted in five random representative images (×200), corrected for lung tissue percentage per image using ImageJ 1.45s software and averaged per animal. Sections were semiquantitatively scored for FoxP3-positive cells as follows: 0, no cells; 1, a few cells; 2, medium amount of cells; and 3, large amount of cells. To calculate the pulmonary FoxP3+/CD3− ratio, we normalized the number of CD3-positive cells in all groups to controls and normalized the relative FoxP3 expression to controls. We then divided the normalized FoxP3 expression by the normalized CD3 expression for FoxP3+/CD3− ratios. Observers were blinded.

**Histology.** Paraffin-embedded RUL tissue sections were stained with hematoxylin and eosin (H&E) and evaluated by two lung pathologists, blinded, to assess lung morphology and presence of neutrophils and lymphocytes.

**Enzyme-linked immunosorbent assay.** Protein concentrations of IL-8 in bronchoalveolar lavage fluid (BALF) and IL-8 and IL-6 in fetal plasma were measured using enzyme-linked immunosorbent assays according to the manufacturer’s instructions, with anti-ovine IL-6 (MAB1004; Merck Millipore, Billerica, MA) or IL-8 (MAB1044; Merck Millipore) as capture antibodies; recombinant ovine IL-6 (6495; ImmunoChemistry Technologies, Bloomington, MN) or IL-8 protein (6542; ImmunoChemistry Technologies) for standards; and anti-ovine IL-6 (AB1839; Merck Millipore) or IL-8 (AB1840; Merck Millipore) as detection antibodies.

**Disaturated phospholipid measurement.** Disaturated phospholipid (DSPL) concentration was determined in BALF as described previously (41). Concentrations were corrected for collected BALF volume, body weight, and left and right lung weight.

**Statistical analysis.** Data are shown as means ± SE. For statistical analysis, GraphPad Prism version 6.01 software was used. To compare groups, the nonparametric Kruskal-Wallis test with Dunn’s Multiple Comparison Test was used. In case of a potential outlier, we tried to identify causes for aberrant results. If no obvious cause was found, the potential outlier was not excluded since it might be a legitimate observation and due to the occurrence of an extreme value at the end of a distribution (7). Instead we used nonparametric methods to analyze our data. These methods make weaker assumptions about underlying distributions than normal-theory methods, thereby minimizing the effect of potential outliers on the overall results (7, 38).

Considering the relative low number of animals per group, we have depicted the actual P values in Figs. 1–4.

**RESULTS**

**Pulmonary inflammation.** H&E-stained slides were evaluated for influx of inflammatory cells. LPS exposure caused influx of neutrophils and lymphocytes compared with controls. In IL-2-treated animals no increase of neutrophils or lymphocytes was observed. Prophylactic IL-2 treatment reduced the infiltrated lymphocytes, whereas the presence of neutrophils within alveolar tissues was not diminished (data not shown).

To characterize the different inflammatory cell subsets, lung slides were stained for CD3, FoxP3, MPO, and PU.1. Intra-amniotic LPS exposure increased the number of CD3-positive cells (P = 0.0168), which was prevented by prophylactic IL-2 treatment (P = 0.0062; Fig. 1, A–D). IL-2 treatment did not change the number of CD3-positive cells compared with control animals (Fig. 1D). Exposure to IL-2, LPS, or the combination led to an increased amount of FoxP3-positive cells compared with controls (P = 0.0292, 0.0027, or 0.0187; Fig. 1, E and G–J). The FoxP3+/CD3− ratio increased after exposure to IL-2 (P = 0.0400) or the combination of IL-2 with LPS (P = 0.0083) compared with controls (Fig. 1F). The FoxP3+/CD3− ratio in the combined treatment group was also higher compared with the LPS group (P = 0.0227).

Numbers of MPO- and PU.1-positive cells were increased after LPS exposure compared with control animals (P = 0.0090 or 0.0496; Fig. 2, A and B). Prophylactic IL-2 treatment in these LPS-exposed animals did not prevent increased MPO- and PU.1-expressing cell numbers (Fig. 2, A and B). Similarly, elevated cell numbers were present in the combined group compared with control (P = 0.0273 or 0.0127; Fig. 2, C–D and E–F) and IL-2 (P = 0.0022 or 0.0455) animals.

IL-8 protein concentrations in BALF are used as a prognostic marker for lung pathologies of preterm infants, including chronic lung disease (25). IL-2 treatment did not affect IL-8 levels in BALF, whereas LPS exposure increased the IL-8 concentration compared with control (P = 0.0227; Fig. 3A). Prophylactic IL-2 treatment did not prevent this increase.

**Systemic inflammation.** Although BALF IL-8 levels were changed, plasma IL-8 levels did not differ between groups at 24 h or 7 days post-LPS or saline exposure (Fig. 3, B and C).

We measured IL-6 protein levels in fetal plasma at 24 h and 3, 5, and 7 days after intra-amniotic LPS or saline to assess a systemic fetal inflammatory response (11). Twenty-four hours after intra-amniotic LPS exposure, IL-6 plasma concentration increased (P = 0.0057), which was not prevented by prophylactic IL-2 treatment (Fig. 3D). At 3, 5, and 7 days after intra-amniotic LPS exposure no increase of plasma IL-6 concentrations was detected (data not shown).

**Morphology.** Morphology was equivalent to the saccular/alveolar phase of lung development (20) and similar between groups according to two independent lung pathologists.

**Lung gas volumes and disaturated phospholipids.** IL-2 treatment increased the lung gas volume compared with control animals (P values ranged from 0.0856 at 10 mmHg to 0.0656 at 40 mmHg; Fig. 4A). LPS (P values ranged between 0.0015 at 0 mmHg and 0.0008 at 40 mmHg) and the combination of IL-2 with LPS (P values ranged from 0.0020 at 0 mmHg to 0.0010 at 40 mmHg) increased lung gas volumes compared with controls.

In addition, we determined the concentration of DSPLs in the BALF. In controls, the concentration of DSPLs was below

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Fig. 1. Characterization of CD3 (A–D)– and FoxP3 (E)–positive cells and their ratio (F) after exposure to lipopolysaccharide (LPS), interleukin (IL)-2, or the combination in the ovine fetal lungs. Shown are representative images of CD3-positive cells for control (A), LPS-treated (B), and IL-2 + LPS-treated (C) animals and representative images of FoxP3-positive cells for control (G), IL-2-treated (H), LPS-treated (I), and IL-2 + LPS-treated (J) animals. Black arrowheads indicate FoxP3-positive cells (H–J). Scale bars: 100 μm. For statistical analysis the Kruskal-Wallis test was performed followed by the Dunn’s Multiple Comparison Test. Animal nos./group are as follows: control n = 6, IL-2 n = 6, LPS n = 6, IL-2 + LPS n = 7 for CD3; control n = 4, IL-2 n = 5, LPS n = 6, IL-2 + LPS n = 6 for FoxP3 and the FoxP3+/CD3+ ratio.
the detection limit (Fig. 4B). IL-2 administration increased the DSPL concentration \((P = 0.0754)\). Increased DSPL values were detected in LPS-exposed animals with or without IL-2 treatment \((P = 0.0007\) or 0.0004\). Combined exposure also increased DSPLs compared with IL-2 alone \((P = 0.0269)\).

**DISCUSSION**

Inadequate resolution of inflammatory responses in infants exposed to chorioamnionitis increases the risk for adverse outcomes of the lung, including BPD (32), which emphasizes the importance of the regulation of pulmonary inflammation in the course of adverse fetal lung development. Because Tregs are important in regulating inflammatory responses and IL-2 is necessary for clonal expansion of Tregs (27, 31, 44, 45), we evaluated the effects of IL-2-driven selective Treg expansion on the fetal lung in an ovine chorioamnionitis model.

IL-2 treatment preferentially expanded Tregs by increasing the FoxP3+\(\)/CD3+\(\) ratio in the fetal lungs. FoxP3 expression was restricted to infiltrated immune cells, which is inconsistent with earlier reports which showed that bronchial epithelial cells can express FoxP3 (2, 8). FoxP3 expression might eventually be too low to be detected in bronchiolar epithelium by an immunohistochemical staining on our fetal ovine tissue. D’Alessio et al. (6) demonstrated in vivo that Tregs are crucial in resolving LPS-induced lung inflammation. Interestingly, the increased FoxP3+\(\)/CD3+\(\) ratio in our model was associated with a moderate immunomodulatory effect in utero. Although prophylactic IL-2 administration did prevent influx of pulmonary T cells following LPS exposure, it was not accompanied by inhibition of systemic immune activation, indicating that this immunomodulatory effect in the lung was not systemically driven. The scope of this study was limited to the fetal lung in utero and not the long-term postnatal outcome. Chorioamnionitis is identified as a risk factor for adverse pulmonary outcome, such as the development of BPD. BPD is a multifactorial disease in which disturbed immune regulation is
considered to be an essential underlying cause (4, 32). Therefore, we can only speculate about the consequences of the enhanced FoxP3+/CD3+ ratio in utero for the long-term postnatal outcome. Previously, it was shown that antenatal LPS exposure enhanced subsequent inflammatory responses in ventilated preterm lungs. Accordingly, mild ventilation-induced indicators of injury were amplified in fetal lambs exposed to LPS prenatally (13). In the present study we did not observe morphological changes. The absence of such changes post-LPS exposure is in line with previous findings in which no morphological differences were found by computerized morphometric analyses (5), which are apparently not provoked in our study since animals were not ventilated (43, 47). Future studies will clarify whether enhancement of the pulmonary FoxP3+/CD3+ ratio prenatally will protect the lungs after birth. Postnatally, these vulnerable preterm infants are exposed to additional injurious events such as mechanical ventilation and sepsis, which are known to induce pulmonary and systemic inflammatory responses, including circulatory IL-8 levels (1, 10, 26, 29).

We for the first time show that systemic IL-2 treatment improved lung gas volume and DSPLs in utero. These bene-

Fig. 3. Bronchoalveolar lavage fluid (BALF) interleukin (IL)-8 levels (A) and plasma IL-8 (B and C) or IL-6 (D) levels in the ovine fetus after exposure to IL-2 with or without lipopolysaccharide (LPS). Protein levels were measured 7 days (A and B) or 24 h (C and D) after intra-amniotic saline or LPS exposure. For statistical analyses, an arbitrary value of 1 pg/ml was assigned to values below the detection limit. Statistical analysis was performed by a Kruskal-Wallis test and Dunn’s Multiple Comparison Test. Animal nos./group are as follows: control n = 4, IL-2 n = 6, LPS n = 5, IL-2 + LPS n = 7 for BALF IL-8; control n = 4, IL-2 n = 6, LPS n = 5, IL-2 + LPS n = 6 for plasma IL-8 7 days; control n = 5, IL-2 n = 6, LPS n = 6, IL-2 + LPS n = 6 for plasma IL-8 and IL-6 24 h.

Fig. 4. Pulmonary pressure-volume curve (A) and disaturated phospholipid concentration (B) of the ovine fetus after exposure to interleukin (IL)-2, lipopolysaccharide (LPS), or the combination. P values are compared with the control group for 40 mmHg pressure (A). For statistical analyses, an arbitrary value of 1 μg/kg was assigned to values below the detection limit (B). The Kruskal-Wallis test was performed followed by the Dunn’s Multiple Comparison Test. Animal nos./group are as follows: control n = 7, IL-2 n = 5, LPS n = 5, IL-2 + LPS n = 6 for the pressure-volume curve; control n = 6, IL-2 n = 6, LPS n = 4, IL-2 + LPS n = 6 for the disaturated phospholipid concentration. BW, body wt.
ficial effects on lung function maintained despite moderate immunomodulatory effects of IL-2 pretreatment in LPS-exposed animals. Because surfactant deficiency in premature infants predisposes to RDS (35), the surprising finding that IL-2 treatment alone increased surfactant lipids and lung gas volume in utero warrants further evaluation to also elucidate the possible mechanism. Notably, IL-2Rα (CD25) distribution in the fetal ovine lung was restricted to pulmonary T cells (data not shown), suggesting that the epithelium responds indirectly to IL-2. However, we cannot rule out the possibility that CD25 expression in the respiratory epithelium is too low to detect by immunohistochemistry, since Lesur et al. (24) demonstrated CD25 protein and RNA transcript expression besides functionality of IL-2 receptors in primary cultures of rat type II cells.

Given the relative small animal numbers per group, we report actual P values and tend to interpret P values between 0.05 and 0.1 as biologically relevant. This assumption will decrease the chance of a false negative finding but increases the chance that one of these differences is a false positive result. Another limitation is that we can only study pulmonary changes in utero at a fixed time point. Nevertheless, we chose this species since ovine pulmonary development closely resembles human lung development (48), and the model enables us to perform complex interventions necessary to administer IL-2 treatment in utero. Furthermore, we cannot elucidate whether the increased number of pulmonary FoxP3-positive cells is the result of proliferation, migration, or a combination of these processes.

In conclusion, systemic IL-2 treatment in utero preferentially expanded Tregs and improved lung gas volume and DSPLs. These beneficial effects on pulmonary function were maintained despite the moderate immuno-modulatory effects of IL-2 treatment in the setting of chorioamnionitis. Combined with the clinical potential of IL-2 in the fetal gut (30) and the fact that no adverse outcomes of IL-2 treatment were observed (such as vascular leakage syndrome, assessed by total protein in the BALF and absence of hypotension), IL-2 may be beneficial in the clinic where antenatal inflammation is often measured despite the moderate immunomodulatory effects of IL-2 pretreatment in LPS-exposed lambs.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

AUTHOR CONTRIBUTIONS

M.G.W., B.W.K., and T.G.W. conception and design of research; M.G.W., D.R.O., M.N., R.K.J., and A.B. performed experiments; M.G.W. and T.D. analyzed data; M.G.W., M.N., B.W.K., and T.G.W. interpreted results of experiments; M.G.W. prepared figures; M.G.W. and T.G.W. drafted manuscript; M.G.W., B.W.K., and T.G.W. edited and revised manuscript; M.G.W., D.R.O., M.N., R.K.J., A.B., T.D., B.W.K., and T.G.W. approved final version of manuscript.

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