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Thymic changes after chorioamnionitis induced by intraamniotic LPS in fetal sheep

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Abstract

Background—T_{reg} mediates homeostasis of the immune system and differentiate under the control of the transcription factor FoxP3 in the fetal thymus.

Objective—We asked if fetal inflammation caused by chorioamnionitis would modulate thymus development.

Methods—Fetal sheep were exposed to an intraamniotic injection (IA) of 10 mg LPS 5h, 1d, 2d or 5d before delivery at 123d gestation days. Cord blood lymphocytes, plasma cortisol and thymus weight were measured. Glucocorticoid receptor-, activated caspase-3-, Ki67-, PCNA-, NF-κB- and FoxP3-positive cells were immunohistochemically evaluated in thymus.

Results—IA LPS decreased the number of circulating lymphocytes by 40% after 1d. Thymus-to-body weight ratios were reduced in all LPS groups by a maximum of 40% at 5d. LPS modestly increased plasma cortisol concentration, increased NF-κB immunostaining in fetal thymus and reduced the number of FoxP3-positive cells by 60% at 1d.

Conclusion—Intraamniotic exposure to LPS induced thymic changes and influenced thymic FoxP3 expression.

Keywords

preterm; fetal inflammatory syndrome; immune development; T lymphocytes; FoxP3; T_{reg}

Introduction

T lymphocyte development and selection in the thymus is a complex process that integrates endogenous and exogenous stimuli during fetal and postnatal life¹. There may be a “window of opportunity/vulnerability” in fetal life where a variety of factors, including infection may modulate the development of the immune system¹. With fetal stress, the thymus can rapidly involute and decrease in size probably because of glucocorticoids-induced apoptosis of immature thymocytes²⁻⁴. Recovery from these processes may be important for the normal development and function of the immune system later in life⁵.

Histologic chorioamnionitis is present in 60% of very preterm deliveries⁶. Many fetal organs may be affected by exposure to antenatal inflammation, which suggests that chorioamnionitis causes a “multi-organ disease of the fetus”⁷⁻¹¹. Fetal thymic involution is associated with the fetal inflammatory response syndrome (FIRS) in women with preterm labor and rupture of membranes¹². Clinical and experimental data indicate that exposure to antenatal inflammation can have both risk and benefit for the fetus¹³. For example chorioamnionitis can induce lung maturation but also may cause brain and gut injury^{11, 14, 15}. The unifying link between the affected organs may be the fetal immune response⁷. Little is known about the effect of preterm birth and chorioamnionitis on T lymphocyte development in the thymus. However, prematurity and chorioamnionitis are associated with increased early childhood wheezing and physician-diagnosed asthma¹⁶. We previously showed in a surgical model of chorioamnionitis that LPS contact with the fetal respiratory tract or gut changed the percentage of CD4, CD8 and increased CD4/CD25 positive cells in the thymus^{11, 17}.

Natural regulatory T lymphocytes (T_{reg}), a subgroup of T lymphocytes, regulate immune responses and participate in tolerance responses¹⁸. T_{reg} are also involved in human diseases such as cancer, infections, autoimmunity, allergy and asthma^{19, 20}. The transcription factor FoxP3 is a marker for T_{reg} in mice and humans²¹. In the mouse, natural FoxP3⁺ T_{reg} are formed in the thymus and contribute to peripheral T_{reg}²². Proinflammatory cytokines and microbial pathogens initiate innate immune responses by NF-κB signaling. The physiological function of NF-κB in adaptive immune pathways is less clear. However, NF-κB is essential for thymocyte development and activation^{23, 24}.

For this study we tested the hypothesis that chorioamnionitis caused by LPS in fetal sheep would modulate changes in T_{reg} lymphocytes in the thymus. We measured cortisol concentrations and lymphocyte counts in the systemic circulation, thymus weight, apoptosis and proliferation, expression of NF-κB, Foxp3 and glucocorticoids receptor in the fetal thymus.

Materials and methods

Animal-model and sample collection

The animal studies were performed in Western Australia using date-mated Merino ewes with singleton pregnancies. All studies were approved by the animal care and use committees at the DAFWA (Department of Agriculture and Food, Western Australia) and at the Cincinnati Children’s Hospital Medical Center, Ohio, USA. In groups of four to seven animals chorioamnionitis was induced 5h, 1d, 2d or 5d before caesarean delivery at 123 days gestation (term 150 days) by intra-amniotic (IA) injection of 10 mg LPS (*Escherichia coli* 055:B5; Sigma, St. Louis MO) (Figure 1). N=7 animals were used for the 5h time point, n=6 animals for the 1d time point, n=4 for the 2 d time point and n=6 animals for the 5d time point. The control group received intraamniotic saline injections at either 5h (n=2), 1d (n=2), 2d (n=2) and 5d (n=2) before delivery. All animals were studied at the same gestational age. Thus we used the composite control. There were no differences between the control animals that received saline injections at different time points before delivery (data not shown). We have therefore

combined the animals as one control group. Results for lung inflammation and maturation associated with chorioamnionitis were previously reported^{25, 26}. Cord blood samples were collected at delivery for complete white blood counts²⁵. Fetal plasma cortisol concentrations were measured using a commercial radioimmunoassay kit (RIA; ICN, Irvine, CA). The inter- and intra-assay coefficients of variation were 0.159 and 0.112. The chest was opened and the intrathoracic thymus was removed. The thymus was weighed, snap frozen, and fixed in formalin.

Immunohistochemistry

Sections (2 μ m, transverse) were cut from formalin fixed, paraffin embedded thymus, and were mounted on APES-(3-amino-propyltriethoxy-silane; Roth, Karlsruhe, Germany) coated slides. Slides were dewaxed and dehydrated in ethanol. Antigen-retrieval was performed by boiling slides for 10 min in citric acid buffer, pH 6.0, using a microwave (750 W). Endogenous peroxidase activity was inhibited by 3% hydrogen peroxide in methanol. PBS with 5% goat serum was used to block non-specific binding. Slides were incubated overnight at 4°C in a humidified chamber with primary antibodies at appropriate dilution in PBS. Caspase-3 activation, via cleavage of its regulatory NH₂-terminal domain, is a well-characterized biochemical marker of apoptosis²⁷. Antibodies against proliferating cell nuclear antigen (PCNA), a nonhistone protein that is elevated in the S, G₂, and M phases of mitosis, and Ki-67, expressed in the S, G₁, G₂, and M phases of mitosis, were used as proliferation-associated antibodies²⁸. The following primary antibodies were used: (i) polyclonal rabbit anti-glucocorticoid receptor (GR) α and β (diluted 1:250, sc-8992, Santa Cruz Biotechnology), (ii) monoclonal rabbit anti-activated caspase-3 (AC-3 diluted 1:100; #9664, Cell Signaling Technology), (iii) monoclonal mouse anti-Ki-67 (diluted: 1: 50; Dako M7240), (iv) monoclonal mouse anti-PCNA (diluted: 1:400; Dako M0879), (v) polyclonal anti-human RelBp68 (component of NF- κ B; diluted: 1:1000; sc-226, Santa Cruz Biotechnology) and (vi) monoclonal mouse anti-FoxP3 (diluted 1:400, 14-7979-82, NatuTec, Frankfurt, Germany). Detection was with an anti-rabbit (GR and AC-3) or an anti-mouse (Ki-67, PCNA, FoxP3) secondary antibodies conjugated to horseradish peroxidase (HRP; Dako Glostrup, Denmark) and developed with 3,3' diaminobenzidine (DAB; brown colour). After counterstaining with hematoxylin, samples were dehydrated and coverslipped. Omission of the primary antibody acted as a negative control. An average for each sample was calculated from five fields that were chosen at random. Numeric analysis was done for apoptotic cells and proliferative cells. Six high-power fields were photographed for the medulla and cortex. The intensity of staining was measured using a semiquantitative scale (magnification $\times$ 200) as no staining (0), weak (+1), moderate (+2), or strong (+3) by a blinded examiner or results are expressed as percentages of all cells counted.

Statistical Analysis

Results are given as means $\pm$ standard error of mean. Comparisons between the groups were performed by analysis of variance (ANOVA) with Student-Newman test as post hoc analysis. Significance was accepted at $p < 0.05$.

Results

Lymphocytes in cord blood and thymus weight

Blood lymphocyte count and thymus weight are markers for stress-induced thymus involution⁴. In this model of IA exposure to LPS, blood lymphocytes were lower than control by 40% after 1d and elevated at 5d (Figure 2A). The thymus/body weight ratios were lower in all LPS exposed groups compared to control (Figure 2B).

Plasma cortisol and expression of corticosteroid receptors in thymus

At 2d after LPS, plasma cortisol levels ($1.3 \pm 0.3 \mu\text{g/dL}$) were higher than control ($0.5 \pm 0.1 \mu\text{g/dL}$) (Figure 3). Immunohistochemistry for the glucocorticoid receptor was performed to evaluate if the small increase in cortisol altered expression of the corticosteroid receptor in the thymus. No differences in immunostaining of the glucocorticoid receptor were detected between the controls and the LPS-exposed groups in the thymic cortex (Figure 3B) and in the thymic medulla (data not shown). The distribution of immunostaining was also not different (data not shown).

Apoptosis and proliferation in thymus

No significant differences in activated caspase-3 immunostaining in the thymus were detected between the control and LPS groups (Figure 4). The percentages of proliferating cells in the thymus, as indicated by Ki-67 (Figure 5A-C) or PCNA (Figure 5D) immunostaining were not different between groups.

Activation of NF- κ B signalling in thymus

The NF- κ B-positive nuclei in the thymic cortex were 2-fold higher than control in the 1d LPS group. A representative section from the control group is shown in Figure 6A and from the 1d LPS group in Figure 6B. The results are summarized in Figure 6C. No distinct staining for NF- κ B-positive cells could be detected in the thymic medulla of the control or LPS groups (data not shown).

Intra-amniotic LPS decreased FoxP3 expression in thymus

The percentage of FoxP3 positive cells in the thymic cortex was lower than control in the 1d LPS group. A representative section from the control group is shown in Figure 7A and from the 1d LPS group in Figure 7B. The results are summarized in Figure 7C.

Comment

Chorioamnionitis induced involution of the fetal thymus, as indicated by activation of NF- κ B in the fetal thymus, the reduced thymic weight, the changed number of circulating lymphocytes and of FoxP3⁺ positive thymocytes. The thymic involution was partially reversible by 5d. These findings of acute thymic involution may be, at least in part, the result of lymphocyte migration into the blood and inflamed organs such as the lung because increase of cell death or apoptosis was not detected.

Decreased blood lymphocytes might be an early sign of acute thymus involution, as a response of the animal to acute stress. Glavina-Durdov et al.⁴ examined acute thymic involution in deceased neonates. The percentage of lymphocytes in the white blood cell count was depending on the grade of thymus involution. There was an increase in blood lymphocytes for grade 0 (resting state) to grade 2 and a decrease in lymphocytes for grade 3 to grade 4. The reduction of lymphocytes in the 1d group of LPS exposed fetuses may reflect the thymic involution. Similarly, the largest changes in Foxp3 expression and NF- κ signalling were found in the 1d LPS exposed group. Normalisation of the Foxp3 expression and the NF- κ signalling together with the increase in blood lymphocytes in the 5d group may imply recovery from thymic involution. The relationship between lymphocyte numbers in peripheral blood and thymic involution were described before²⁹⁻³². In fetal sheep lymphocytes increased with LPS exposure in lung and amniotic fluid²⁶.

The role of glucocorticoids in thymocyte homeostasis is controversial. Mouse models genetically modified for glucocorticoid receptor expression or function yield conflicting results in terms of the effects of cortisol on thymocytes. Glucocorticoids can not only limit thymocyte

numbers by the induction of apoptosis, but may also promote the survival and proliferation of these cells in young mice³³. There was a small increase in plasma cortisol in our study and no increase in numbers of apoptotic lymphocytes in the thymus. Thymic involution occurred before the small increase in plasma cortisol occurred, suggesting another mechanism causing the involution.

Thymic involution by fetal stress has been associated with the development of bronchopulmonary dysplasia (BPD) in preterm infants³⁴. A small thymus detected at birth on the routine chest radiograph was predictive for the development of BPD in very low birth weight preterm infants³⁵, suggesting that immune mechanisms that contribute to lung injury and BPD begin antenatally. Rosen *et al.* demonstrated that thymic architecture and T lymphocyte function were altered with BPD and suggested that autoreactive T lymphocytes could contribute to lung injury³⁴. There is also an association between a markedly decreased thymic size - measured on chest radiography or ultrasound at birth - and subclinical chorioamnionitis in very low birth weight infants³⁶⁻³⁹. These clinical studies used radiological methods to estimate thymic size and there are only a few reports of thymus morphology in neonates based on post-mortem findings. Toti *et al.* found that thymic weight was decreased in spontaneously aborted fetuses exposed to extensive chorioamnionitis⁴⁰. There also was a reduced corticomedullary ratio, a significant change in the relationship between thymic parenchyma and thymic interstitial tissue with resulting increased organ complexity, a reduction of thymocytes and other degenerative processes such as monocytes/macrophage infiltration of Hassall's bodies⁴⁰. These findings may represent the most severe form of a fetal systemic inflammatory response syndrome resulting in fetal death. Our finding of thymic weight reduction was much less marked. Also the histological change of thymus described by Toti *et al.* was not seen in this fetal sheep model. Furthermore there were no changes in markers of apoptosis and proliferation in the thymocytes and only transient effects on NF- κ B activation and FoxP3 expression were detected. The decreased number of Foxp3-positive thymocytes could contribute to pathogenesis of BPD if the chronic inflammation were ineffectively controlled or suppressed^{41, 42}. A possible explanation for the differences between the clinical studies and our findings could be that the fetal sheep were exposed once to LPS and the human fetuses were exposed to a prolonged infection^{30, 31}. However, in clinical practice most of the cases of intra-amniotic infections are asymptomatic⁴³. This mild chorioamnionitis may have consequences for both the mother and her fetus, such as endometritis, preterm birth or increased morbidity and mortality for the newborn^{44, 45}.

Numerous reports suggest that the transfer of foreign antigens from the mother (including self antigens, food antigens, and antigens associated with infectious agents carried by the mother) to the fetus are a common occurrence⁴⁶⁻⁴⁸. Billingham and colleagues first advanced the concept that "actively acquired immunologic tolerance" occurs as a result of fetal exposure to such foreign antigens⁴⁹. As the immune system develops, T cells must be selected or regulated to become tolerant of self antigens and reactive against foreign antigens. The induction of such tolerance is thought to be attributable to the generation of T_{regs} that suppress fetal immune responses. Mold *et al.*⁴⁸ found recently that substantial numbers of maternal cells cross the placenta, inducing the T_{regs} that suppress fetal anti-maternal immunity and persist at least until early adulthood. This form of antigen-specific tolerance is induced *in utero* and probably contributes to immune-response regulation after birth⁴⁸. We previously found, in this sheep model of chorioamnionitis induced by LPS, profound changes in the function of cord blood monocytes and pulmonary monocytes⁵⁰⁻⁵². We also found a tolerance of monocytic cells to a second stimulation with the same LPS preparation both *in vitro* and *in vivo*. This tolerance was not limited to LPS as the agonist, but affected also other Toll-like receptor agonists that signal through the same second messenger pathways^{52, 53}. Apparently, the fetus is able to tolerate and/or control inflammation *in utero* given the low incidence of early onset sepsis⁵³.

The importance of inflammatory exposure on the developing thymus in children has been highlighted by epidemiological studies. Exposure of infants to animals in a farming environment early in life can protect the child from development of hay fever and atopic sensitisation⁵⁴. Exposure in early life has a much stronger effect as compared to exposure occurring after the first year of life⁵⁵. Moreover, maternal exposure to animal sheds during pregnancy resulted in protection from allergy in children to school age⁵⁵. There does appear to be a “window of opportunity/vulnerability” in fetal/early life where a variety of factors, including infection and inflammation induced by chorioamnionitis may be important for the development of the immune system.

Limitations of this study are the limited sample sizes and the single fetal LPS exposure. Human fetuses exposed to chorioamnionitis may have prolonged exposure to proinflammatory mediators. The timing for interactions of bacteria with the fetal immune system is not well understood⁵³. Results from a single fetal LPS exposure may differ from the human situation where there is a polymicrobial infection of the amniotic cavity⁵⁶. Furthermore, the evaluation of the Foxp3 expression in thymus only by immunohistochemistry limits the interpretation of the results. The activation of NF- κ B signalling in the thymus could affect thymocyte development, especially T_{reg} differentiation and activation^{57, 58}. The decrease in FoxP3-positive thymocytes may result from NF- κ B activation^{57, 58}. We tried unsuccessfully to double immunostain for Foxp3 and NF- κ B in thymus.

An advantage of this animal model is the possibility of monitoring thymic involution. The thymic involution was partially reversible. Therefore, the neonatal thymus and immune system may have the plasticity to recover from stress and/or inflammation *in utero*. Possible consequences of these temporary changes in the immune system in postnatal life remain to be studied, but accumulating evidence suggest an important role for antenatal inflammation in fetal programming and subsequent disease development^{59, 60}.

Clinical Implications

This study suggests that antenatal inflammation during fetal life induces thymus changes and influences T_{reg} development and immune system may have the plasticity to recover from stress and/or inflammation *in utero*. Possible consequences of these temporary changes in the immune system in postnatal life remain to be studied, but accumulating evidence suggests an important role for antenatal inflammation in fetal programming and subsequent disease development.

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Figure 1.

Study design. Four to seven animals per group received LPS 10 mg or NaCl 0.9% (control); 5h, 1d, 2d or 5d after treatment the lambs were delivered and evaluated.

Figure 2.

Quantification of blood lymphocytes (A) and thymus weight (B). A: Number of lymphocytes in complete white blood cell counts from cord blood samples at delivery (*p<0.05 vs. control). B: Thymus/body ratio was lower than control after intraamniotic LPS injection at 5h, 1d, 2d or 5d (*p<0.05 vs. control).

Figure 3.

A: Quantification of cortisol in plasma. B: quantification of glucocorticoid receptor immunostaining in the fetal thymus cortex (*p < 0.05 versus control).

Figure 4.

Quantification of apoptosis in fetal thymus with staining for caspase-3. Evaluation of caspase-3 expression in thymus in control group (A) and in LPS group (1d) (B) by immunohistochemistry. Representative sections for each time point are shown for caspase-3 (A+B). Magnification 200x. C. Quantification of caspase-3-positive thymocytes in thymus sections by immunohistochemistry.

Figure 5. Quantification of cell proliferation in fetal thymus with staining for Ki-67 (A-C) and PCNA (D). Evaluation of Ki-67 expression in thymus in control group (A) and in LPS group (B) by immunohistochemistry. Representative sections for each time point are shown. Magnification 200x. C. Quantification of Ki-67 (C) and caspase-3-positive (D) thymocytes in thymus sections by immunohistochemistry.

Figure 6.

Detection of NF-κB-positive thymocytes in cortex of thymus in control group (A) and in LPS group (B) by immunohistochemistry. Representative sections for each time point are shown. Magnification 200x. C. Quantification of NF-κB-positive thymocytes in thymus sections identified by immunohistochemistry. (* $p < 0.05$ vs. controls).

Figure 7. Evaluation of FoxP3 expression in cortex of thymus in control group (A) and in LPS group (B) by immunohistochemistry. Representative sections for each time point are shown. Magnification 200x. C. Quantification of FoxP3-positive thymocytes in thymus sections by immunohistochemistry (* $p < 0.05$ vs. controls).