



Single early prenatal lipopolysaccharide exposure prevents subsequent airway inflammation response in an experimental model of asthma

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ABSTRACT

Aims: There has been emerging interest in the prenatal determinants of respiratory disease. In utero factors have been reported to play a role in airway development, inflammation, and remodeling. Specifically, prenatal exposure to endotoxins might regulate tolerance to allergens later in life. The present study investigated whether prenatal lipopolysaccharide (LPS) administration alters subsequent offspring allergen-induced inflammatory response in adult rats.

Main methods: Pregnant Wistar rats were treated with LPS (100 µg/kg, i.p.) on gestation day 9.5 and their ovariectomized female offspring were sensitized and challenged with OVA later in adulthood. The bronchoalveolar lavage (BAL) fluid, peripheral blood, bone marrow leukocytes and passive cutaneous anaphylaxis were evaluated in these 75-day-old pups.

Key findings: OVA sensitized pups of NaCl treated rats showed an increase of leucocytes in BAL after OVA challenge. This increase was attenuated, when mothers were exposed to a single LPS injection early in pregnancy. Thus, LPS prenatal treatment resulted in (1) lower increased total and differential (macrophages, neutrophils, eosinophils and lymphocytes) BAL cellularity count; (2) increased number of total, mononuclear and polymorphonuclear cells in the peripheral blood; and (3) no differences in bone marrow cellularity or passive cutaneous anaphylaxis.

Significance: In conclusion, female pups treated prenatally with LPS presented an attenuated response to experimentally-induced asthma. We observed reduced immune cell migration from peripheral blood to the lungs, with no effect on the production of bone marrow cells or antibodies. It was suggested that inflammatory events such as exposure to LPS in early fetal life can attenuate allergic inflammation in the lung, which is a common symptom in asthma.

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Introduction

Asthma is an inflammatory lung disease characterized by cellular recruitment, plasma exudation and airway hyper-responsiveness. These hallmarks of asthma are induced by inflammatory mediators, which are released from sensitized mast cells after antigen challenge (Bryce et al., 2006; Busse and Lemanske, 2001).

The so-called 'hygiene hypothesis' or microbial deprivation hypothesis suggests that a lack of exposure to microbial stimulation early in childhood is a major factor involved in the increasing prevalence of allergy and asthma (Conrad et al., 2009; Kalliomaki et al., 2010). Although traditional postnatal 'hygiene hypothesis' risk

factors play a clear role in the development of asthma and atopic disease, there has been emerging interest in the prenatal determinants of this respiratory disease (Kumar, 2008). There may be in utero and early-life factors that play a role in airway development, inflammation, and remodeling (Kumar, 2008). Specifically, it has been found that a number of the epidemiological protective factors of the so-called 'hygiene hypothesis' are related to the effects of endotoxin and other microbial exposure (Braun-Fahrlander et al., 2002; Gehring et al., 2002) via innate immune system activation (Gern et al., 2004). For example, in utero exposure to farming environments elicited protective effects on asthma phenotypes (Douwes et al., 2008). Furthermore, it has been reported that prenatal exposure to immunological factors may be even more important than early-life exposures (Douwes et al., 2008; Kumar, 2008).

In fact, contact with lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria, in early infancy is associated with decreased allergen sensitization (Gerhold et al., 2002; Wang and McCusker, 2006).

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As far as we know, all studies performed on prenatal LPS exposure and asthma have been performed in late pregnancy, or throughout pregnancy (Blumer et al., 2005; Cao et al., 2010; Datti et al., 2008). Thus, we believed that it would be relevant to study the effects of a single early prenatal LPS exposure on gestation day (GD) 9.5 on allergic lung inflammatory response in adult female rats. To this end, ovariectomized female pups were then sensitized and challenged with ovalbumin (OVA) in adulthood. The bronchoalveolar lavage (BAL) fluid, peripheral blood, bone marrow leukocytes and passive cutaneous anaphylaxis were evaluated in these pups. GD 9.5 was selected for LPS exposure since this date coincides with the beginning of the pivotal pseudoglandular stage of prenatal lung development in rats. This stage is most likely involved with the susceptibility to developing asthma in later life (Walters et al., 1987; Warburton et al., 2000).

Materials and methods

Animals

Pregnant Wistar rats from our own colony, weighing 216–263 g each, were used (GD 0 = spermatozoa in the vaginal smear). Dams were individually housed in polypropylene cages (38×32×6 cm) at controlled room temperature (22±2 °C), humidity (65–70%), and artificial lighting (12-hour light/12-hour dark cycle, lights on at 6:00 a.m.) with free access to Nuvelab® rodent chow (Nuvital Co., São Paulo, SP, Brazil) and filtered water. Sterilized and residue-free wood shavings were used as animal bedding. The dams were randomly distributed into two control groups and one experimental group (n=10/each group). These rats were allowed to give birth and nurture their offspring normally. No cross-fostering procedure was used. The day of birth was considered as postnatal day (PND) 1. No handling was performed on PND 1, but on PND 2, 8 offspring (4 males and 4 females) were randomly selected. Litters smaller than 8 pups were culled. These eight pups were kept with each dam until weaning (PND 21). On PND 21, the littermates were separated, housed together by sex and grouped in the same laboratory conditions as their parents. One female pup of each litter was used for the tests in adulthood; the male offspring were separated, to be used in other experiments (Kirsten et al., 2010b). All experiments were performed between 7:00 and 12:00 am. The animals used in this study were kept in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources of the School of Veterinary Medicine, University of São Paulo, Brazil (protocol No. 925/2006, FMVZ-USP). These guidelines are similar to those of the National Institutes of Health, Bethesda, MD. Experiments were carried out in accordance with the GLP protocols and with quality assurance methods.

Treatment

LPS (from *Escherichia coli*, Sigma®, serotype 0127: B8) was dissolved in sterile saline (50 µg/ml of LPS in 0.9% NaCl solution) and was administered intraperitoneally (i.p.) to pregnant rats at a dose of 100 µg/kg on GD 9.5. This dose was chosen because it has been reported to (1) elicit sickness behavior in the dams, (2) induce endocrine alterations, (3) increase cytokines at the placental level, and (4) impair offspring viability and reduce the social behavior of male offspring during infancy and adulthood (Kirsten et al., 2010b; Spencer et al., 2007; Wang et al., 2006). The control group consisted of pregnant rats submitted to the same treatment schedule, but with sterile saline (0.9% NaCl). Each dam was treated with 0.1 ml/100 g saline solution. An additional control group of ovariectomized female rats were used as a negative control group, here called basal group. These animals were not submitted to any treatment, i.e. prenatal and OVA-sensitized and challenged in adulthood.

Ovariectomy and validation of ovariectomy

Twenty female rat pups (1 from each litter, n=10/each group) were ovariectomized on PND 53 to minimize the variables of the experiment, since female sex hormones modify the course of allergic pulmonary inflammation (Riffo-Vasquez et al., 2007). The ovariectomy and validation of ovariectomy were performed as described elsewhere (Ligeiro de Oliveira et al., 2004). Briefly, animals were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg) by i.p. injection. Upon laparotomy, the ovaries were surgically removed. The effectiveness of ovariectomy was determined by analysis of the vaginal smear and by quantification of uterine weight. Only successfully ovariectomized females in the diestrus phase were used in the experiments.

Rat model of allergic lung inflammation

The rat model of allergic lung inflammation was performed as described elsewhere (Ligeiro de Oliveira et al., 2008). Briefly, after 7 days of ovariectomy (PND 60), the female pups were sensitized with OVA (Egg Albumin Grade II, Sigma Chemical Company® USA). Seven days later (PND 67), the animals were boosted with OVA, and seven days later (PND 74), challenged with OVA aerosol.

Bronchoalveolar lavage (BAL) fluid analysis

On PND 75, BAL was performed in female pups as previously described by (Ligeiro de Oliveira et al., 2008). Briefly, the animals were anesthetized with ketamine plus xylazine, sacrificed, and their lungs were flushed for the total leukocyte and differential cell counts.

Peripheral blood analysis

Immediately before BAL collection, blood samples were taken from the animal's abdominal aorta. The total number of cells and the differential leukocyte counts were performed as described elsewhere (Ligeiro de Oliveira et al., 2008).

Bone marrow analysis

The total number of bone marrow cells was quantified in the femoral marrow lavage (FML) fluid obtained as described elsewhere (Ligeiro de Oliveira et al., 2008). Briefly, after BAL and peripheral blood collections, rat femurs were removed, the cells were collected and analyzed for total leukocyte counts.

Passive cutaneous anaphylaxis

Passive cutaneous anaphylaxis (PCA) reactions were performed as described elsewhere (Ligeiro de Oliveira et al., 2004). Briefly, sera from sensitized female pups were serially diluted and injected in the shaved dorsal skin of unsensitized naive rats. After 24 h, these animals received OVA and Evans blue in saline. Thirty minutes later, the rats were killed, the skin was removed and the diameter of the dye stain on the inner surface of the skin was measured. The PCA (IgE) titers represent the highest dilution of the serum that gave a dye stain >5 mm in diameter.

Statistical analysis

Results were expressed as mean ± SEM. One female rat pup from each dam was considered in the offspring studies to avoid litter effects. Homoscedasticity was verified through the F test. Normality was verified through the Kolmogorov–Smirnov test. Thus, parametric data were analyzed by Student's *t*-test or by ANOVA, followed by the

multiple comparisons Tukey–Kramer test. In all cases results were considered significant if $p < 0.05$.

Results

Fig. 1 shows the effects of prenatal LPS exposure on total and differential BAL cell count in OVA-sensitized and -challenged female rats. This experiment was conducted to characterize OVA-induced allergic inflammation in rats treated with prenatal LPS or vehicle. The total number of cells (Panel A) in the BAL of control animals challenged with OVA was higher than that of the basal group ($p < 0.001$); rat pups treated prenatally with LPS and challenged with OVA did show a smaller increase in the number of cells in BAL after OVA challenge, compared to control/OVA group ($p < 0.001$); however, the total number of cells in the LPS/OVA group remained higher than that observed in the basal group ($p < 0.001$). The number of macrophages (Panel B) of the control/OVA group was higher than those of the basal group ($p < 0.001$); a significant reduction was observed in the LPS/OVA group in relation to the control/OVA group ($p < 0.05$) but not between the basal and LPS/OVA groups ($p > 0.05$). The

number of neutrophils (Panel C) in the control/OVA group was increased in comparison to the basal group ($p < 0.001$); the LPS/OVA group presented a decrease in these cell types in relation to the control/OVA group ($p < 0.001$) but not in relation to the basal group ($p > 0.05$). The number of lymphocytes (Panel D) was increased in control/OVA group data as compared to those of the basal group ($p < 0.01$); a significant decrease in the number of these cells was observed in the LPS/OVA group when compared to the control/OVA group ($p < 0.05$); no difference was found between LPS/OVA group and the basal group ($p > 0.05$). Panel E represents the number of eosinophils. Because the basal data were null, the unpaired t -test with Welch correction was applied to compare the data of control/OVA and LPS/OVA groups. Thus, the LPS/OVA group presented a significant decrease in the number of eosinophils, compared to the control/OVA group ($p < 0.0001$).

Because the number of cells in the lungs resulting from bronchoprovocation was lowered increased by prenatal treatment with LPS, we quantified (1) total number of, (2) mononuclear and (3) polymorphonuclear cells present in the peripheral blood of prenatally treated and control female rats sensitized and challenged during adulthood with OVA

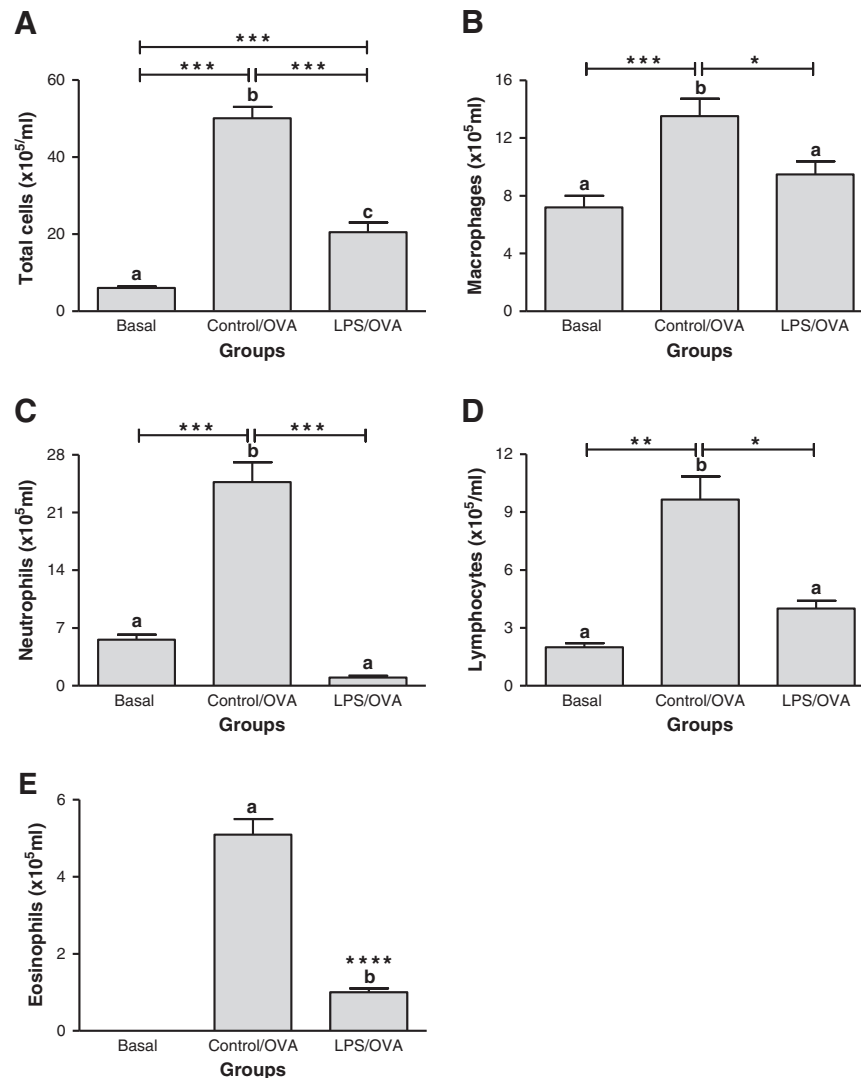


Fig. 1. Effects of prenatal LPS exposure (100 µg/kg on GD 9.5) on BAL (A) total cells (B) macrophages, (C) neutrophils, (D) lymphocytes and (E) eosinophils of OVA-sensitized and challenged female rats. Basal group: naïve animals that remained undisturbed, with no treatment; Control/OVA: rats prenatally exposed to 0.9% NaCl solution and OVA-sensitized on PND 60; LPS/OVA: rats prenatally exposed to LPS and OVA-sensitized on PND 60. All measures were performed 12 h after the OVA challenge. Values are presented as mean \pm SEM. $n = 10$ for both groups. Different superscript letters mean statistically significant differences and the same letters mean no significant differences. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ (ANOVA and Tukey–Kramer tests).

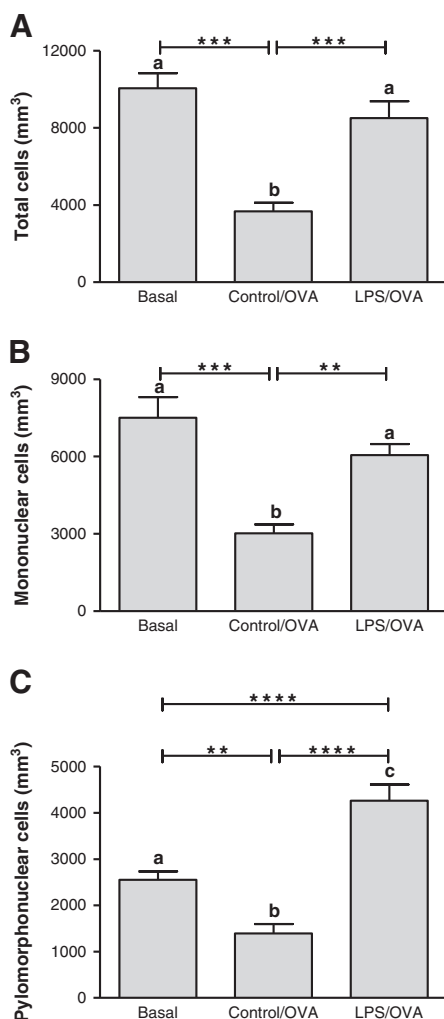


Fig. 2. Effects of prenatal LPS exposure (100 µg/kg on GD 9.5) on (A) total number, (B) mononuclear and (C) polymorphonuclear cells in the peripheral blood of OVA-sensitized and challenged female rats. Basal group: naïve animals that remained undisturbed, with no treatment; Control/OVA: rats prenatally exposed to 0.9% NaCl solution and OVA-sensitized on PND 60; LPS/OVA: rats prenatally exposed to LPS and OVA-sensitized on PND 60. All measures were performed 12 h after the OVA challenge. Values are presented as mean ± SEM. n = 10 for both groups. Different superscripts letters mean statistically significant differences and the same letters mean no significant differences. ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ (ANOVA and Tukey–Kramer tests).

(Fig. 2). In relation to the total number of cells (Panel A) the control/OVA group presented a smaller number of cells in the blood than that measured in both basal ($p < 0.001$) and LPS/OVA ($p < 0.001$) groups; no differences were detected between basal and LPS/OVA groups ($p > 0.05$). Regarding the mononuclear cells (Panel B) there was a decrease in the control/OVA group as compared to the basal group ($p < 0.001$); the LPS/OVA group presented a significant increase in relation to the control/OVA group ($p < 0.01$) but not in relation to the basal group ($p > 0.05$). Concerning the circulating polymorphonuclear cells (Panel C) there was a decrease in the control/OVA group in relation to those of the basal group ($p < 0.01$); a significant increase was found in the LPS/OVA group compared to the both control group/OVA ($p < 0.0001$) and basal group ($p < 0.0001$).

No significant differences were observed between groups with regard to the bone marrow analysis (basal group: 40.20 ± 3.80 ; control/OVA group: 44.70 ± 3.60 ; LPS/OVA group: 51.20 ± 3.20 ; $p > 0.05$).

PCA levels in naïve rats that received serum from OVA-sensitized female pups (prenatally treated with LPS or vehicle on GD9.5) were

identical in both groups (basal group: 12.80 ± 1.96 ; control/OVA group: 12.80 ± 1.96 ; LPS/OVA group: 12.80 ± 1.96 ; $p > 0.05$).

Discussion

The increased levels of total and differential cells located in lung (BAL) in the control/OVA group versus basal group indicate that the lung allergic response was correctly induced in the rats. Moreover, female rats prenatally treated with LPS and challenged with OVA as compared to the control/OVA group presented a lower increase in total and differential cells located in lung. Thus, prenatal LPS prevented cell migration to the lung during the allergen response. Furthermore, in the control/OVA group as compared to the basal group, the number of total, mononuclear and polymorphonuclear cells in the peripheral blood was decreased. This decrease is considered normal and occurs due to cellular migration to the lungs (Ligeiro de Oliveira et al., 2008). However, females prenatally treated with LPS did not present a decrease in these parameters, suggesting that peripheral immune cells did not migrate to lung, probably due to mobilization impairment. Notably, the mobilization, activation and trafficking of effector cells to the airway are controlled by a complex pleiotropic cytokine milieu derived from resident airway cells including epithelial and airway smooth muscle cells (Chung, 2001). It has been shown that several substances induce pro-inflammatory cytokines production. In this context, LPS exposure increases pro-inflammatory cytokine levels, such as tumor necrosis factor- α , interleukin-1, and interleukin-6 in the airway (Oyoshi et al., 2007). Thus, LPS might have been responsible for that mobilization impairment.

With regard to bone marrow cellularity, no differences were observed in rats prenatally treated with LPS, then sensitized and challenged with OVA in adulthood, as compared to the control/OVA group. Thus, hematopoiesis (processing, maturation and lymphocyte differentiation) seems not modified by prenatal LPS treatment on GD 9.5. However, it should not be forgotten that the quantification of the total cell number in the bone marrow does not exclude possible alterations on cell maturation or differentiation phases, or on cell functional or morphological modifications. These are facts that are now under investigation.

Moreover, the lack of differences between the groups in the PCA analysis suggests that a mechanism other than the production of antibodies was responsible for the LPS-mediated long-term improvement of OVA sensitization effects in rats. To further characterize the allergic response, we measured the OVA-specific IgE using PCA as an index. Significant differences were not observed for the levels of OVA-specific IgE in the LPS group when compared to the control group.

Besides the previous findings of our group related to behavioral impairments (play behavior and adulthood social interaction) in rat pups prenatally treated with LPS (100 µg/kg on GD 9.5) (Kirsten et al., 2010b), the present study revealed changes in the immune system status of these pups. Specifically, prenatally LPS-treated females presented during adulthood with attenuated experimental lung allergic response, i.e., reduced lung inflammation, a common symptom of asthma (Bryce et al., 2006; Busse and Lemanske, 2001).

It is felt that the mechanisms responsible for the impairments presently found and reported in the prenatally LPS-treated female rat pups do not directly involve LPS; this endotoxin was not found in pups after a prenatal injection (Ashdown et al., 2006). It has been suggested that the effects of maternal LPS exposure on the developing fetus are mediated by cytokine induction within the maternal circulation and placenta (Ashdown et al., 2006; Cai et al., 2000; Smith et al., 2007; Urakubo et al., 2001).

The possibility of using bacterial compounds during gestation in order to prevent allergic diseases has been studied for some time. Prenatal LPS (aerosolized) exposure has been shown to prevent subsequent allergen sensitization in offspring through inhibition of T-helper type (Th) 2 immune responses (Gerhold et al., 2006). However,

the prenatal treatment of this latter study was too long (3× per week, GD 7 until delivery) and the effects were only found when prenatal treatment was combined with further postnatal LPS exposure (Gerhold et al., 2006). The present study revealed similar results, but with a single exposure to LPS on GD 9.5 and without postnatal LPS exposure. This fact strongly reinforces the relevance of an inflammatory insult on the plasticity of lung allergy during the prenatal period.

Similarly, Blumer et al. (2005) found that a subchronic exposure to LPS (female BALB/c mice received three injections of LPS on days 5, 3 and 1 prior to mating and during every third day during the gestation period) modulate the development of allergies in the offspring.

The reason why rats prenatally treated with LPS presented attenuated cell migration to lungs could be explained by the 'hygiene hypothesis'. Shifts in the Th1/Th2 immune system balance seem to fit well with the so-called 'hygiene hypothesis'. Normally, the immune system of animals with allergic diseases such as asthma is inclined towards Th2-type responses. It is known that animals prenatally treated with LPS present high levels of pro-inflammatory cytokines, i.e., increased Th1 response (Pavlov and Tracey, 2004). Therefore, the activation of innate immunity is expected to normalize the Th1 and Th2 immune system balance and to suppress the excessive reaction of Th2-type responses (Yoshida et al., 2009).

Incidentally, we speculate that the prenatal LPS treatment on GD 9.5 employed here was effective in suppressing allergen-induced immune airway responses, interfering with the traffic and/or recruitment of Th2 cells to the sites of inflammation.

Conclusion

Although the results indicate a beneficial effect for prenatal LPS treatment, since female rats presented an attenuated allergic reaction, studies in other fields are needed to validate this treatment as a prophylactic approach to asthma. In fact, rats subjected to this treatment also presented several behavioral impairments (Kirsten et al., 2010a; Kirsten et al., 2010b). However, immunomodulation with bacterial compounds during the gestation period has also been suggested as a possible effective mode for first-step primary prevention of allergic diseases. Furthermore, the optimal time frames for initiating immunomodulation to elicit a sufficient effect against allergen sensitization are still unclear. These findings may contribute to understanding the genesis and treatment of allergic responses, such as asthma. Bacterial exposure on GD 9.5 in rats may be considered for studies of strategies to prevent the development of allergic diseases.

Conflict of interest statement

The authors declare there are no conflicts of interest.

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