



Prenatal exposure to endotoxin in rats attenuates the allergic airways eosinophil infiltration in the adult offspring: Role of inducible nitric oxide synthase activation

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Abstract

A correlation between stressful events experienced by the mother during pregnancy and progression of respiratory disease in descendants has been reported. Prenatal exposure to lipopolysaccharide (LPS) reduces allergic airway inflammation in mice offspring. In this study we investigated whether reduction of airways inflammation by maternal LPS exposure involves activation of inducible nitric oxide synthase (iNOS) at prenatal life. Since LPS also induces the release of TNF- α , and that this cytokine has been implicated in pathogenesis of asthma, we also evaluated whether TNF- α plays a role in the allergic airways inflammation by maternal LPS exposure. Pregnant rats were pretreated with the iNOS inhibitor aminoguanidine (50 mg/rat per day; given from day 13 of gestation up to delivery) before exposure to LPS (7 μ g/kg, given at day 17 of gestation). At adult ages, female and male offspring were sensitized with ovalbumin (OVA), and 14 days later they were challenged with this allergen. OVA challenge in sensitized offspring increased markedly the eosinophil number in bronchoalveolar lavage (BAL) fluid at 48 h compared with the non-sensitized group. However, the eosinophil number was largely reduced in offspring from maternal LPS exposure, irrespective of offspring gender. Maternal pretreatment with aminoguanidine fully reversed the eosinophil suppression by LPS. The maternal LPS exposure also reduced the eosinophil number in bone marrow and peripheral blood of offspring, but this was not affected by aminoguanidine. No differences in TNF- α levels in BAL fluid were found. In conclusion, our study shows that maternal LPS exposure markedly reduces allergic airways eosinophil recruitment in adult offspring by mechanisms possibly involving iNOS activation.

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1. Introduction

Asthma is a chronic inflammatory condition in the airways characterized by accumulation of mucus in the lumen of the airway, edema in bronchial mucosa and increased bronchial responsiveness. Besides activation of mast cells, macrophages and T lymphocytes in the airway mucosa, eosinophil infiltration into the airways plays a key role in the pathogenesis of asthma [1,2]. Correlations between eosinophil number and the severity of this disease have also been reported [3]. Evidence suggests

that recruitment of eosinophils into sites of inflammation is a multifactorial and multistep process, involving eosinophil–endothelial interactions through adhesion molecules and local generation of chemotactic agents that direct cell migration into the inflamed airways [4]. Studies attempting to understand the role of the eosinophil in the pathophysiology of bronchial asthma are therefore a focus of intense interest.

Clinical studies suggested a correlation between stressful events lived by the mother during pregnancy and progression of respiratory disease in the descendants [5–7]. Epidemiological studies have shown that children that live in rural environments and consequently are exposed to immune stimuli are highly resistant to allergic diseases, including asthma [8,9]. The level of environmental

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exposure to endotoxin and other bacterial wall components has been accepted as an important protective determinant for the development of atopic diseases in childhood [10].

Nitric oxide (NO) is a reactive oxygen species involved in the regulation of immune responses, and participates in the diverse type of acute and chronic inflammatory responses, including bronchial asthma [11]. NO is synthesized by the so-called NO synthases (NOS), which exist as constitutive (cNOS) and inducible (iNOS) isoforms. Whilst the former are normally present in cells regulating physiological functions, the iNOS isoform is expressed upon appropriate inflammatory stimulation by for example lipopolysaccharide (LPS) or cytokines [12]. Nitric oxide (NO) is one important signaling molecules involved in LPS-induced responses [13]. Recent reports showed that prenatal LPS exposure in mice (associated or not with postnatal LPS exposure) prevents allergen-induced airway inflammation in the adult offspring, suggesting that LPS may already operate in prenatal life in order to modulate the development of allergies in the offspring [14,15]. Therefore, the present study was designed to investigate whether the reduction of airways inflammation by maternal LPS exposure takes place by iNOS-dependent mechanisms. To achieve this, we treated pregnant rats with the selective iNOS inhibitor aminoguanidine (day 13 of gestation up to delivery) before exposure to LPS (day 17 of gestation), and studied the allergen-induced airway cell recruitment in the female and male offspring at adult ages. Since LPS activates immune responses and stimulates mononuclear cells to release TNF- α [16], and that this cytokine has been implicated in pathogenesis of asthma in animals and humans [17–20], we also evaluated the TNF- α levels in bronchoalveolar lavage (BAL) fluid in both female and male offspring.

2. Materials and methods

2.1. Animals

Female nulliparous Wistar rats (200–230 g) provided by the Biology Institute Laboratory Animal Center (CEMIB) of the State University of Campinas (UNICAMP) were used. They were individually housed at $26 \pm 2^\circ\text{C}$ with food and water ad libitum on a 12 h light:dark cycle with the lights turned on at 6:00 a.m. The experimental protocols were approved by the Ethical Principles in Animal Research adopted by the Brazilian College for Animal Experimentation (COBEA).

The males were placed in female cage. The date of conception (day 0 of pregnancy) was determined when sperm was detected in expelled vaginal plugs. At birth, litters were reduced to eight pups per dam, four females and four males, when possible. The offspring were kept together with their dams and left undisturbed, except for routine care. On weaning, at the age of 21 days, the animals were housed in a group of 10 according to the prenatal treatment until testing at adult age (75 days). The pups were weighed at birth and weaning. Tests were performed using one to two rats per litter per test.

2.2. Prenatal treatments and experimental protocols with the adult offspring

On the day 17 of gestation (third phase of pregnancy), rats received a subcutaneous injection of LPS (from *E. coli*; Sigma, St. Louis, USA) at the dose of $7\mu\text{g/kg}$, whereas control pregnant rats received saline (SAL; 1.0 ml/kg). This dose of LPS caused no abortion and/or resorption (higher doses of LPS were not employed because in preliminary experiments LPS caused approximately 50% with $10\mu\text{g/kg}$, and 100% abortion for 100 and $350\mu\text{g/kg}$).

In order to evaluate whether iNOS mediates the LPS-induced responses, separate groups of pregnant rats were pretreated with the iNOS inhibitor aminoguanidine given in the drinking water (1 mg/ml) from day 13 to labor, according to a previous study [21]. The concentration of aminoguanidine in the water gave a total amount of 50 mg/rat per day .

At adult ages (75 days), female and male offspring were each divided into four groups: maternal saline offspring group (litters from saline-injected dams), maternal LPS offspring group (litters from LPS-treated dams), maternal aminoguanidine/LPS offspring group (litters from aminoguanidine/LPS-treated dams) and maternal aminoguanidine offspring group (litters from aminoguanidine-treated dams). Each of these groups was further divided into another two groups: Ovalbumin (OVA)-sensitized offspring (rats sensitized with OVA) and non-sensitized offspring (rats that received OVA vehicle; see details below). On day 14 after OVA sensitization, all the offspring were intratracheally injected with either OVA or saline. BAL was then performed at 48 h after OVA instillation, a time by which a large eosinophil infiltration into the airways of OVA-sensitized rats can be detected. Leucocyte counts of the BAL fluid were then done. Cell number was also concomitantly evaluated in the peripheral blood and bone marrow, as detailed below.

2.3. Allergen-induced airway inflammation protocol

Active sensitization against OVA (grade III OVA chicken egg; Sigma, St. Louis, USA) was performed by s.c. injection of 0.15 ml suspension containing $200\mu\text{g}$ of OVA and 8 mg Al(OH)_3 prepared in saline. Non-sensitized offspring received only 8 mg Al(OH)_3 . On day 14 after sensitization, both sensitized and non-sensitized offspring were anaesthetized with halothane and exsanguinated. The trachea was exposed through a midline ventral incision of approximately 0.5 cm length in the neck. With the aid of a 26.5 gauge needle, 0.4 ml of 0.25% solution of OVA was injected into the airways. Immediately after this procedure, the animals were sutured and allowed to recover from anaesthesia. BAL was performed 48 h after OVA challenge. Briefly, the trachea was exposed and cannulated with a polyethylene tube (1 mm diameter) connected to a syringe. The lungs were washed by flushing with PBS solution containing heparin (20 UI/ml). The PBS buffer was instilled through the tracheal cannula as one 10-ml aliquot

followed by three 5-ml aliquots. The fluid recovered after each aliquot instillation (approximately 20 ml) was combined and centrifuged ($1000 \times g$ for 10 min at 20°C). The cell supernatant was discarded and the cell pellet was resuspended in 2 ml of PBS solution. Total cell counts were carried out using a Newbauer chamber, while differential counts were carried out on a minimum of 200 cells using cytopsin preparations stained with Diff-Quick[®]. The cells were classified as neutrophils, eosinophils, mast cells, lymphocytes and macrophages based on normal morphological criteria.

2.4. Leukocyte counts in peripheral blood and bone marrow

Blood samples were obtained from the abdominal artery before BAL procedure. Total cell counts were carried in Newbauer chamber, while differential counts were carried out on blood smears stained with Diff-Quick[®] (Behring Ag, Düringen, Switzerland). The cells were classified as neutrophils, eosinophils, and mononuclear based on normal morphological criteria. For measurement of cells in bone marrow, femurs were removed from rats immediately after killing. The epiphyses were cut transversely and bone marrow cells were flushed out with PBS containing heparin (20 UI/ml). A differential count was carried out on a minimum of 200 cells using cytopsin preparation stained with Diff-Quick[®]. Results are expressed as the number of leukocytes per femur. Cells were classified as neutrophils, eosinophils and mononuclear cells based on normal morphological criteria. Eosinophils were evaluated as the mixture of mature and immature forms recognized by the intensely eosinophilic granules.

2.5. Determination of tumor necrosis factor- α (TNF- α) levels in BAL

The TNF- α levels were measured in BAL fluid at 48 h post-OVA challenge using commercially available enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions TNF- α (R & D Systems, Minneapolis, MN, USA).

2.6. Statistical analysis

Data are presented as the mean values $\pm$ sem and were analyzed by analysis of variance (ANOVA) for multiple comparisons followed by Bonferroni test or Student's unpaired *t*-test where appropriate. In both cases, the level of significance was set at $p < 0.05$.

3. Results

3.1. Body weight

To evaluate the weight development, female and male pups were weighed immediately after birth (day 0) and on the weaning (day 21). Table 1 shows that prenatal exposure to LPS enhanced significantly ($p < 0.05$) the body weight of

the male and female pups, both at birth (10% and 10% increase, respectively) and weaning (22% and 19% increase, respectively) when compared to saline group.

3.2. Leukocyte counts in BAL fluid of adult offspring

We have designed experiments to evaluate if prenatal LPS exposure affects the allergic pulmonary inflammation in the female and male adult offspring. Briefly, non-sensitized and OVA-sensitized offspring were intratracheally injected with OVA, and 48 h thereafter the leukocyte recruitment in BAL fluid was evaluated.

The BAL fluid from non-sensitized offspring contained $2.9 \pm 0.7 \times 10^6$ and $3.1 \pm 0.5 \times 10^6$ leucocytes/BAL for female and male offspring, respectively. The cells were virtually all (about 99%) mononuclear cells with eosinophils absent. As expected, the OVA challenge in previously sensitized rats (maternal saline exposure) caused a marked increase in eosinophils in BAL fluid in comparison with non-sensitized group, as observed in both female and male offspring (Fig. 1). However, the eosinophil number was largely reduced ($p < 0.05$) in female and male offspring from the maternal LPS exposure group in comparison with the saline group. In such animals, the maternal pretreatment with the iNOS inhibitor aminoguanidine prevented the cell suppression by LPS in the adult offspring (Fig. 1). Aminoguanidine alone had no significant effect on eosinophil counts. The number of neutrophils in BAL fluid of adult offspring was not significantly affected in any studied group (Table 2). The number of mononuclear cells in BAL fluid of adult offspring was reduced by maternal LPS exposure in comparison with saline group; however, maternal aminoguanidine treatment did not significantly affect the reduction by LPS compared with aminoguanidine alone (Table 2).

3.3. Leukocyte counts in bone marrow and peripheral blood

In order to explore whether eosinophil reduction in BAL fluid of adult offspring by maternal LPS exposure reflects modifications at the level of bone marrow and peripheral blood, the counts of leukocytes in both of these compartments were evaluated in all groups. Fig. 2A shows that

Table 1

Body weight (g) of rat pups at birth (day 0) and weaning (day 21) from pregnant rats treated with saline (SAL) or lipopolysaccharide (LPS; 7 $\mu\text{g/kg}$)

Day	Female pups		Male pups	
	Birth	Weaning	Birth	Weaning
SAL	5.7 ± 0.1	41.9 ± 1.5	6.0 ± 0.1	43.3 ± 1.0
LPS	$6.3 \pm 0.1^{**}$	$49.9 \pm 0.7^{***}$	$6.6 \pm 0.1^*$	$53.1 \pm 1.0^{***}$

* $p < 0.05$ compared with SAL.

** $p < 0.01$ compared with SAL.

*** $p < 0.001$ compared with SAL.

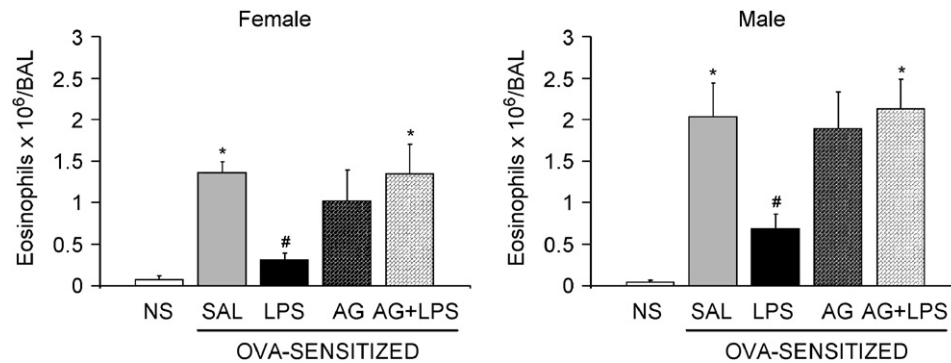


Fig. 1. Number of eosinophils in bronchoalveolar lavage (BAL) fluid of adult female and male offspring. Pregnant rats were pretreated with the inducible nitric oxide inhibitor aminoguanidine (AG; 50 mg/rat per day; given from day 13 of gestation up to delivery) before exposure to lipopolysaccharide (LPS; 7 μ g/kg, given at day 17 of gestation) or saline (SAL). At adult ages, female and males were sensitized with ovalbumin (OVA), and 14 days later they were intratracheally challenged with OVA. At 48 h post-OVA challenge, the eosinophil counts in BAL were evaluated. Data from each non-sensitized (NS) group (SAL, LPS, AG and AG + LPS) were pooled because no differences between each other were found. Results are the mean $\pm$ SEM for $n = 20$ (NS) or $n = 5$ (OVA-sensitized groups). * $p < 0.05$ compared with NS; # $p < 0.05$ compared with SAL, AG or AG + LPS.

Table 2

Counts of neutrophils and mononuclear cells in bronchoalveolar lavage (BAL) fluid of non-sensitized and OVA-sensitized adult female and male offspring from pregnant rats pretreated with aminoguanidine prior exposure to lipopolysaccharide or saline

	Female		Male	
	Neutrophils	Mononuclear	Neutrophils	Mononuclear
NS	0.05 $\pm$ 0.02	2.4 $\pm$ 0.3	0.02 $\pm$ 0.01	2.9 $\pm$ 0.4
OVA-SAL	0.04 $\pm$ 0.01	3.4 $\pm$ 0.3	0.02 $\pm$ 0.01	3.1 $\pm$ 0.5
OVA-LPS	0.04 $\pm$ 0.01	2.2 $\pm$ 0.6*	0.05 $\pm$ 0.02	1.5 $\pm$ 0.2*
OVA-AG	0.05 $\pm$ 0.01	3.9 $\pm$ 0.6	0.06 $\pm$ 0.01	4.5 $\pm$ 0.6
OVA-LPSAG	0.08 $\pm$ 0.02	2.5 $\pm$ 0.4	0.08 $\pm$ 0.02	3.2 $\pm$ 0.6

Pregnant rats treated with aminoguanidine (AG; 50 mg/rat per day; given from day 13 of gestation up to delivery) prior exposure to lipopolysaccharide (LPS; 7 μ g/kg, given at day 17 of gestation) or saline (SAL). Bronchoalveolar lavage (BAL) fluid was collected at 48 h after ovalbumin (OVA) challenge in both non-sensitized and previously sensitized offspring. Counts of neutrophils and mononuclear cells ($\times 10^6$ /BAL) from each non-sensitized (NS) groups (SAL, LPS, AG + LPS and AG) were pooled because no differences between each other were found. Results are the mean $\pm$ SEM for $n = 20$ (NS) or $n = 5$ (OVA-sensitized groups).

* $p < 0.05$ compared with SAL.

OVA challenge in sensitized female offspring (saline group) caused a marked increase ($p < 0.05$) in bone marrow eosinophils compared with non-sensitized rats, and that this was prevented in offspring from maternal LPS exposure. Maternal pretreatment with aminoguanidine did not significantly affect the eosinophil number in comparison with the sensitized animals injected with either saline or LPS (Fig. 2A). The number of neutrophils and mononuclear cells in bone marrow was not significantly altered in any group ($n = 5$ –12; not shown).

In peripheral blood, OVA challenge in sensitized female offspring (saline group) caused a significant increase ($p < 0.05$) in circulating eosinophils compared with non-sensitized rats (Fig. 2B). However, no significant changes in the eosinophil counts were found between OVA-challenged

groups, except for the LPS/aminoguanidine group where a significant reduction in eosinophil counts was observed ($p < 0.05$).

3.4. Levels of TNF- α in BAL fluid

The measurement of TNF- α in BAL fluid at 48 h post OVA challenge revealed negligible or undetectable levels of this cytokine in all groups in both female and male offspring ($n = 5$; not shown).

4. Discussion

In the present study we have showed that maternal LPS exposure in rats reduces the allergen-induced airways eosinophil infiltration in the adult offspring, and that is prevented by pretreatment of pregnant rats with the iNOS inhibitor aminoguanidine.

We have used a single dose of LPS (7 μ g/kg), given subcutaneously on day 17 of gestation. The last third phase of pregnancy in rats (days 14–21 of gestation) is accepted as a critical period where exposure to physical and chemical insults may lead to disorders in the adult descendants [22,23]. Higher doses of LPS ($> 10 \mu$ g/kg) could not be tested because it induced fetal abortion and/or resorption. Interestingly, fetal abortion caused by LPS in mice is prevented by pre-treatment with aminoguanidine indicating that maternal LPS administration exerts a direct effect on trophoblasts by stimulating the induction of iNOS, leading to NO production, and in turn to *in utero* embryo death [24]. A recent study also showed that intraperitoneal administration of LPS (or the NO-donor *S*-nitroso-*N*-acetylpenicillamine) to pregnant mice causes 100% embryonic resorption, an effect significantly reversed by the iNOS inhibitor aminoguanidine [25].

Asthma-like airway inflammation is achieved in rodents by subcutaneous injection of OVA adsorbed in $Al(OH)_3$ that leads to an increased production of IgE antibodies for this allergen. Thus, in OVA-sensitized rats, challenge with

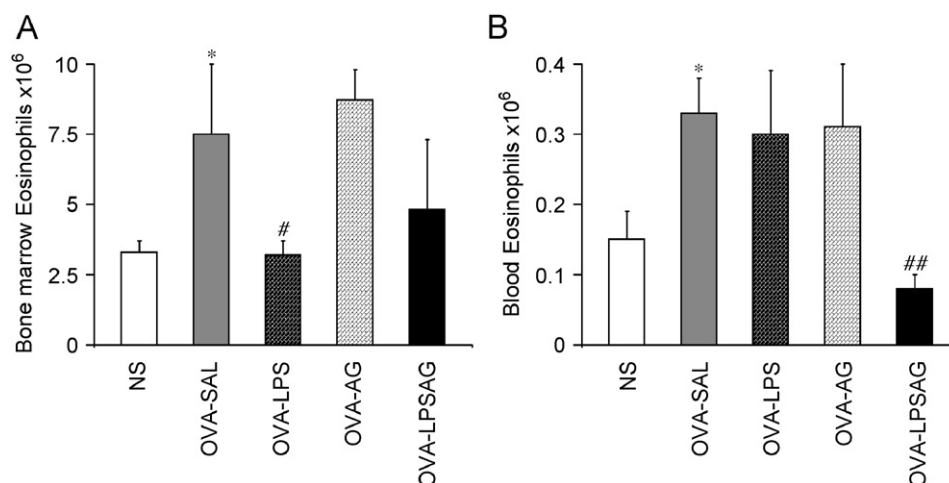


Fig. 2. Eosinophil counts in bone marrow (panel A) and peripheral blood (panel B) of ovalbumin (OVA)-sensitized female offspring. Pregnant rats were pretreated with aminoguanidine (AG; 50 mg/rat per day; given from day 13 of gestation up to delivery) before exposure to lipopolysaccharide (LPS; 7 µg/kg, given at day 17 of gestation) or saline (SAL). Peripheral blood and bone marrow were collected at 48 h after OVA challenge in both non-sensitized and previously sensitized offspring. Data from each non-sensitized (NS) groups (SAL, LPS, AG + LPS and AG) were pooled because no differences between each other were found. Results are the mean $\pm$ SEM for $n = 20$ (NS) or $n = 5$ (OVA-sensitized groups). * $p < 0.05$ compared with NS; # $p < 0.05$ compared with SAL. ## $p < 0.05$ compared with SAL, LPS or LPS + AG.

this allergen evokes an initial recruitment of neutrophils followed by a delayed eosinophil influx that is usually seen at 24–48 h post-OVA challenge [26–28]. Accordingly, in our study, OVA challenge in previously sensitized adult offspring (saline group) resulted in higher number of eosinophils in BAL fluid at 48 h post-challenge. This increased airways eosinophil infiltration in OVA-challenged rats was largely attenuated in the offspring from the maternal LPS exposure group. Furthermore, pretreatment of pregnant rats with the iNOS inhibitor aminoguanidine prevented the effect of LPS on eosinophil number in BAL fluid strongly suggesting that reduction of eosinophil infiltration by maternal LPS exposure takes place by iNOS activation. It is likely therefore that the resulting large NO production in prenatal life influences the allergic airways eosinophil recruitment in the adult offspring. Compared with eosinophils, prenatal LPS treatment reduced to a lesser extent the number of mononuclear cells in BAL fluid, and that was not significantly prevented by maternal aminoguanidine. Although the time-period of 48 h post-OVA challenge is not exactly suitable to evaluate neutrophils, our data showed that neutrophil counts were not significantly affected by the maternal LPS exposure. In general, our data with BAL fluid suggest that iNOS-derived NO at maternal life acts to preferentially regulate eosinophil trafficking at adult life. Although we have used aminoguanidine as a selective iNOS inhibitor, caution should be taken when using this compound because it possesses other pharmacological properties in vivo and in vitro such as inhibition of formation of highly reactive advanced glycosylation end products (AGEs) and diamine oxidase [29].

It is known that antigen challenge also activates a systemic response that provokes inflammatory cell production by the bone marrow [30]. The eosinophils are recruited

from the bone marrow where progenitor cells proliferate and differentiate within the microenvironment in response to cytokine activation [31]; however, the molecular mechanisms leading to mobilization of mature bone marrow eosinophils to peripheral blood and the sequence of events from their activation to recruitment into inflammatory tissue sites are still poorly understood. In our study, an examination of bone marrow in the OVA-sensitized adult offspring (saline group) revealed an increased number of eosinophils at 48 h post-challenge, which was accompanied by a significant elevation of eosinophil number in circulating blood. This increased number of eosinophils in both bone marrow and peripheral blood is consistent with previous studies dealing with OVA-sensitized animal models [32,33] and asthmatic subjects after inhaled antigen [30]. It is accepted that blood eosinophilia reflects the release of bone marrow eosinophils into the circulation. In keeping with the studies with BAL fluid, the increased number of eosinophils in bone marrow was markedly attenuated in the offspring from maternal LPS exposure group, whereas no changes in the neutrophils and mononuclear cells were seen. In contrast to the BAL fluid data, however, the pretreatment of pregnant rats with aminoguanidine neither modified the eosinophil suppression by LPS in bone marrow nor in the peripheral blood. This suggests that iNOS activation and NO production by the maternal LPS exposure modulates the eosinophil trafficking in the offspring at the level of airways, with no involvement at other compartments such as bone marrow and circulating blood.

LPS is known to activate various immune responses and stimulates monocytes/macrophages to release a variety of inflammatory cytokines, including TNF- α [16]. Therefore, we hypothesized that production of TNF- α after OVA challenge could be modified in the offspring from the

maternal LPS exposure group. However, the measurement of TNF- α in BAL fluid at 48 h revealed low levels of this cytokine with no differences among groups, suggesting that TNF- α may not participate in the reduced allergic eosinophil response seen in the LPS-exposed groups. However, in most systems, 48 h is not an optimum time for measurement of TNF- α following allergen challenge. TNF- α is usually detected at earlier times (3 h) after OVA challenge [34], a time by which could not be employed in the present study.

In our study, the female and male offspring from the maternal LPS exposed groups exhibited higher body weight (at birth and weaning), a finding consistent with a previous study [35]. This may reflect the release of vasodilator mediators that enhance placental blood flow and consequently nutrients and oxygen offer. However, it is unlikely that reduction of eosinophil infiltration in BAL fluid in the LPS group is linked to increased body weight because obesity rather increases the risk for respiratory diseases in humans [36].

In conclusion, our present study shows that maternal LPS exposure markedly reduces the eosinophils in the bone marrow to sensitization in offspring. Additionally, the inhibition of iNOS during maternal LPS stimulation increases the ability for eosinophils to be recruited to the lung in offspring by mechanisms unrelated to cell proliferation and differentiation in the bone marrow microenvironment.

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