

Effects of intrauterine exposure to concentrated ambient particles on allergic sensitization in juvenile mice

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ABSTRACT

Intrauterine exposure to particulate matter (PM) has been associated with an increased risk of asthma development, which may differ by the age of asthma onset, sex, and pollutant concentration. To investigate the pulmonary effects of in utero exposure to concentrated urban ambient particles (CAPs) in response to house dust mite (HDM) sensitization in juvenile mice. Mice were exposed to CAPs (600 µg/m³ PM_{2.5}) during the gestational period. Twenty-two-day postnatal mice were sensitized with HDM (100 µg, intranasally, 3 times per week). Airway responsiveness (AHR), serum immunoglobulin, and lung inflammation were assessed after 43 days of the postnatal period. Female (n = 47) and male (n = 43) mice were divided into four groups as follows: (1) FA: not exposed to CAPs; (2) CAPs: exposed to CAPs; (3) HDM: sensitized to HDM; and (4) CAPs+HDM: exposed to CAPs and HDM-sensitized. PM_{2.5} exposure did not worsen lung hyperresponsiveness or allergic inflammation in sensitized animals. The levels of the lung cytokines IL-4, TNF-α, and IL-2 were differentially altered in male and female animals. Males presented hyporesponsiveness and increased lung macrophagic inflammation. There were no epigenetic changes in the IL-4 gene. In conclusion, intrauterine exposure ambient PM_{2.5} did not worsened allergic pulmonary susceptibility but affected the pulmonary immune profile and lung function, which differed by sex.

1. Introduction

Ninety-two percent of the world population lives in cities where air quality exceeds World Health Organization (WHO) guideline limits

(WHO. World Health Organization, 2016). São Paulo is the largest megacity in South America, with more than 12 million inhabitants and an increasing mobile fleet of 8 million vehicles ([IBGE and Instituto Brasileiro de Geografia e Estatística, 2020]). In this city, particulate

Abbreviations: Al, Aluminium; AHR, Airway hyperresponsiveness; AUC, Area under the curve; BALF, Bronchoalveolar lavage fluid; BaP, Benzo[a]pyrene; BbF, Benzo[b]fluoranthene; Ca, Calcium; CAPs, Concentrated ambient particles; DEP, Diesel exhaust particles; ETS, Environmental tobacco smoke; Ers, Elastance/elastic resistance; FA, Filtered air; HDM, House dust mites; Fe, Iron; IBGE, Instituto Brasileiro de Geografia e Estatística; IL, Interleukin; K, Potassium; Mch, Methacholine; Na, Sodium; OVA, Ovalbumin; PAH, Polycyclic aromatic hydrocarbon; PBS, Phosphate-buffered saline; Phe, Phenanthrene; ROFA, Residual oil fly ash; Rrs, Pulmonary resistance; S, Sulfur; Si, Silicon; Th2, T-helper type 2 cells; Th17, T-helper type 17 cells; TNF-α, Tumour necrosis factor-alpha; UFPs, Ultrafine particles; Zn, Zinc; WHO, World Health Organization.

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matter with aerodynamic diameters less than 2.5 micrometres (PM_{2.5}) consistently exceeds the annual 10 µg/m³ levels recommended by the WHO (WHO. World Health Organization, 2016). Nearly 1724 deaths could be avoided, resulting in a gain of US\$ 4.96 billion annually if São Paulo could decrease PM_{2.5} levels by 5 µg/m³ (Abe and Miraglia, 2016).

The adverse effects of air pollution are felt mainly in the susceptible groups such as asthmatic children. Asthmatic children can be adversely affected by air pollution in different ways (Yan et al., 2020). Prenatal and perinatal exposures to air pollution components increase the risk of asthma development during childhood (Lee et al., 2018; Hehua et al., 2017). Exposure to air pollutants also affects asthma outcomes, such as exacerbations, hospitalizations and lung function in children (Tiotiu et al., 2020).

Several experimental studies confirmed this epidemiological evidence. Prenatal or postnatal environmental exposure to PM_{2.5} has been shown to influence lung development (Dolinoy et al., 2007; de Barros et al., 2018d), which may result in increased susceptibility to respiratory diseases in adulthood (Christensen et al., 2017; Ferrini et al., 2017). Experimental research also showed that air pollution exposure has a negative effect on the development of respiratory allergic inflammation in young mice (Zhou et al., 2021).

Intrauterine exposure to air pollutants has been associated with the development of asthma in children, which seems to depend on the age of onset, sex and pollutant concentrations, among others (Sbihi et al., 2016; W. Yan et al., 2020). It is expected that environmental impacts exert more adverse effects at very young ages because of the immaturity in the lung and immune system and rapid growing changes (Lee et al., 2018). Accordingly, prenatal exposure to diesel exhaust particles (DEP) (Fedulov et al., 2008), residual oil fly ash (ROFA) (Hamada et al., 2007) and ozone (Fu et al., 2020) increased asthma susceptibility in mouse pups (day 3 to day 9) sensitized with ovalbumin (OVA) or house dust mites (HDM). On the other hand, low doses of ambient ultrafine particles (UFPs) (Rychlik et al., 2019) during pregnancy suppressed T_H2 immune allergic responses in HDM-sensitized mice at 3 days of age. Exposure to low doses of formaldehyde in pregnant rats suppressed the development of asthma in young adult rats (Fu et al., 2020). Therefore, it is likely that the type of exposure and the age of allergic sensitization affect pulmonary outcomes (Ferrini et al., 2017). The diagnosis of asthma is made with more certainty in older children than in infants, who may present transient wheezing that is due to other causes (Martinez et al., 1995); however, few experimental studies have analysed the effects of PM_{2.5} exposure on juvenile mice.

Epigenetic changes induced by urban PM may be one possible mechanism linked to increased asthma susceptibility in individuals with intrauterine exposure to air pollution (Ji et al., 2016). It is speculated that air pollution-induced DNA methylation may increase the risk of allergic airway disease by silencing the T_H1 pathway (Martino and Prescott, 2011). A balanced and fully functional immune system is important for combating the numerous diseases of childhood and adult life (Wang et al., 2013).

Taken together, there is still room to understand the effects of in utero exposure to ambient PM_{2.5} in the development of allergic diseases and its association with epigenetic changes in young individuals. Therefore, in this study, we examined the effects of intrauterine exposure to ambient levels of PM_{2.5} on the development of pulmonary allergic sensitization in juvenile mice.

2. Methods

2.1. Ethics statement

This study was approved by the Ethical Commission for Animals of the School of Medicine at the University of São Paulo (n°137/15).

2.2. Animals

Specific pathogen-free (SPF) Balb/C young adult male (♂, n = 20) and female mice (♀, n = 20) weighing ~25 g were obtained from the animal facility at the School of Medicine at the University of São Paulo and were housed in ventilated racks with FA (high-efficiency particulate air filter) at constant room temperature (22 °C) with controlled humidity and a 12-h light/dark cycle. Food and water were available *ad libitum*. Animals were mated in a proportion of 1♂:1♀. Mating was evaluated daily by the presence of spermatozooids in the vaginal smear. After pregnancy confirmation, two groups with 10 pregnant mice each were formed: (1) exposed to CAPs (CAPs) (target: 600 µg/m³ PM_{2.5}) and (2) exposed to filtered air (FA).

2.3. Concentrated ambient particles (CAPs) exposure protocol

Mice were intrauterinely exposed during the entire gestational period to concentrated ambient PM_{2.5} using a Harvard ambient particle concentrator (HAPC) located within the main campus of the School of Medicine of the University of São Paulo. The HAPC concentrates particles minor than 2.5 µm at to 30 times (de Barros et al., 2018d; de Oliveira Alves et al., 2020) and the physicochemical characteristics of PM_{2.5} are maintained (de Brito et al., 2014; Sioutas et al., 1995; Yoshizaki et al., 2017). It has two chambers inhalation, one receiving concentrated ambient particles and the other receiving the clean air.

The animals were exposed inside inhalation chambers to filtered air or to concentrated particles (600 µg/m³ PM_{2.5}) simulating an ambient 24-h mean concentration of 25 µg/m³ PM_{2.5}, as in the WHO guideline (WHO. World Health Organization, 2005). To achieve an accumulated dose (concentration vs time product) of 600 µg/m³, the time of the exposure was controlled to ensure the same concentration for all groups (Fig. 1). The concentration of CAPs was monitored in real time using a particulate monitor (DataRam4, Thermo Fisher Scientific) to adjust the concentration precisely during the exposure duration. After birth, the mice and pups were maintained at the animal facility during the 21 days of lactation.

2.4. Sampling and PM_{2.5} elementary characterization

The 24-h concentration of PM_{2.5} was collected with fifteen polycarbonate filters, and trace analysis was performed as previously described by de Oliveira Alves et al. (2020). The elemental composition of PM_{2.5} is shown in Table 1. These compounds are emission markers for heavy vehicles and resuspension of soil at urban centres (de Brito et al., 2014).

Polycyclic aromatic hydrocarbon (PAH) concentrations have been characterized by de Oliveira Alves et al. (2020). Total PAH concentrations were 14 ng/m³, and benzo[b]fluoranthene (BbF), phenanthrene (Phe), and benzo[a]pyrene (BaP) were the most dominant.

2.5. House dust mite (HDM) sensitization

After weaning (PND 21), female (n = 47) and male (n = 43) descendants were separated in specific cages and intranasally sensitized with HDMs (*Dermatophagoides pteronyssinus*, Greer Laboratories, Lenoir, NC, USA) at 100 µg/30 µL of phosphate-buffered saline (PBS) or with saline in the control animals, 3 times per week. Four groups for each sex were formed: FA (not exposed to CAPs, n = 12♂ and 11♀); CAPs (exposed to PM_{2.5}, n = 8♂ and 11♀); HDM (sensitized with HDMs, n = 14♂ and 12♀); and CAPs + HDM (exposed to PM_{2.5} and HDM sensitized, n = 9♂ and 13♀) (Fig. 1). Lung function, bronchoalveolar lavage fluid (BALF), blood serum, and lung tissues were examined on postnatal day 43. Details on the pregnant weight, litter size and weight of offspring can be found in the online supplement (Tables S1 and S2).

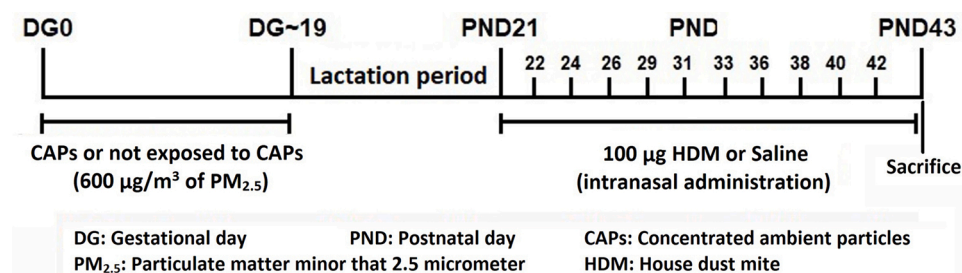


Fig. 1. Experimental study design.

Table 1

Elementary composition of $\text{PM}_{2.5}$ as assessed by an energy dispersive X-ray fluorescence spectrometer. Values are expressed as the mean $\pm$ SD and in micrograms per cubic metre.

Elements ($\mu\text{g}/\text{m}^3$)			
S	.94181	$\pm$	.49824
K	.33876	$\pm$	.24004
Fe	.21654	$\pm$	.14027
Si	.19339	$\pm$	.15806
Zn	.13355	$\pm$	.16458
Na	.12293	$\pm$	.10141
Al	.12003	$\pm$	.10830
Ca	.10560	$\pm$	.07552
P	.03178	$\pm$	.01646
Cl	.02615	$\pm$	.02701
Mg	.01665	$\pm$	.00643
Pb	.01628	$\pm$	.01893
Ti	.01475	$\pm$	.01347
Cu	.01397	$\pm$	.00982
Sb	.00915	$\pm$	.00686
Mn	.00813	$\pm$	.00338
Br	.00494	$\pm$	.00332
Se	.00284	$\pm$	.00165
Sr	.00260	$\pm$	.00247
Ni	.00167	$\pm$	.00101
V	.00165	$\pm$	.00123
As	.00154	$\pm$	.00223
Cr	.00135	$\pm$	.00094
Rb	.00125	$\pm$	.00076

Number of collected filters 15.

2.6. Lung function analysis

Animals were anaesthetized with 50 mg/kg sodium pentobarbital, i. p., tracheostomized and connected to a small ventilator (Harvard 683; Harvard Apparatus Co., South Natick, MA, USA) with a respiratory frequency of 150 times/min and a volume of 10 mL/kg. Tracheal pressure and airflow measurements were taken after crescent concentration administration (3, 30 and 300 mg/mL) of methacholine (Mch) (Ara-ntes-Costa et al., 2014; Timmerman et al., 2019).

2.7. IgE, IgG1, and IgG2a analysis

After the lung function test, blood samples were collected from the vena cava and were then centrifuged (1000 rpm, 4 °C, 20 min) to obtain serum that was stored at -70 °C. The levels of IgE (1110–05; Southern Biotech, USA), IgG1 (1070–05; Southern Biotech, USA) and IgG2a (1080–05; Southern Biotech, USA) were measured in blood serum using ELISA kits according to the manufacturer's recommendations. The colour reaction was read in a spectrophotometer at 490 nm (Brügge-mann et al., 2017).

2.8. Bronchoalveolar lavage fluid (BALF)

Mice were euthanized and tracheostomized, and lungs were washed 3 times with instillations of 0.5 mL of PBS via the tracheal cannula. Total

cell and inflammatory cell analyses were performed as previously described (Timmerman et al., 2019; Vieira et al., 2012).

2.9. Cytokine measurements in BALF

The levels of IL-2, IL-4, IL-5, and IL-17A/F (BioLegend, CA, USA), tumour necrosis factor alpha (TNF- α) and thymic stromal lymphopoietin (TSLP) (RD Systems, MN, USA) were measured in BALF using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's recommendations (Timmerman et al., 2019).

2.10. Histopathological lung analysis

The lungs were removed, fixed by intratracheal instillation of 10 % buffered formalin solution and paraffin embedded after 24 h. Five-micrometre-thick sections were stained with Congo Red for quantification of eosinophil density in the peribronchial space. The macrophages were quantified using immunohistochemical cell staining of slides. The analyses were performed as previously described by Timmerman et al. (2019).

2.11. Methylation analysis

The methylation status of the IL-4 promoter region was evaluated by methylation-specific PCR (MSP-PCR) and direct deoxyribonucleic acid (DNA) sequencing. Briefly, genomic DNA was extracted from mouse lungs using the DNeasy Blood & Tissue Kit (Cat No./ID: 80204, Qiagen) and treated with bisulfite using EpiTect Bisulfite Kits (Cat No./ID: 69504, Qiagen) following the manufacturer's instructions.

The MSP primers for mouse IL-4 (ENSMUST00000140684.7) were designed for the 2000-bp promoter/upstream 5' UTR sequence covering five CpG dinucleotides using the METHPRIMER online primer design tool (<https://www.urogene.org/cgi-bin/methprimer/methprimer.cgi>) (Table 2).

The methylation status was confirmed by Sanger sequencing in an Applied Biosystems™ 3730 DNA Analyzer at the Centro de Sequenciamento do Programa Rede de Equipamentos Multiusuários (PREMiUM) do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo localizado no Centro de Investigação Translacional em Oncologia do Instituto do Câncer do Estado de São Paulo (CTO/ICESP) (<https://www.premium.fm.usp.br>).

2.12. Statistical analysis

The Kolmogorov-Smirnov test was used to check if the data were normally distributed. Lung mechanics data were analysed using the general linear model for repeated measures. Normally distributed variables are reported as the mean $\pm$ SD and were analysed using a multivariate generalized linear model with post hoc comparisons with the Bonferroni test. The non-normally distributed variables are reported as the median and interquartile range (IQR: 25–75 %) and were analysed using the Kruskal-Wallis test. Statistical analyses were performed using SPSS 21.0. The level of significance was set to 5%.

Table 2
Primer sequence.

Primer	Primer sequence	Amplicon size	Annealing temperature
meth_foward	TAAAAATTATTGAGAGAGATTATCGG	277 bp	55 °C
meth_reverse	CAACAAAACAAAAAACATCG		
unmeth_foward	TAAAAATTATTGAGAGAGATTATTGG		
unmeth_reverse	CAACAAAACAAAAAACATCACT		

3. Results

3.1. Effects of gestational exposure to CAPs on fertility and foetal growth

The effects of intrauterine PM_{2.5} exposure were determined by comparing the fertility, gestational length, weight at pregnancy, litter size, and sex ratio between the control and CAPs groups. No differences were observed between the groups (see Table S3, online supplement).

3.2. Effects of allergic sensitization: HDM versus saline groups

Independent of sex and CAPs exposure, HDM-sensitized animals presented an increase in the following parameters: (1) Rrs in the dose-response curve ($p = 0.001$) (Fig. 2, A–C) and maximum MCh response ($p = 0.004$) (Fig. 2, D); (2) levels of serum IgG1 and IgG2a ($p = 0.000$) (Fig. 3, A and B); (3) peribronchiolar eosinophil density ($p = 0.002$) (Fig. 4); and (4) BALF IL-5 ($p = 0.000$), IL-17 ($p = 0.010$), and TSLP levels ($p = 0.033$) (Fig. 5, C, D, and F).

3.3. Effect of exposure: CAPs versus FA groups

CAPs induced a reduction in BALF IL-4 levels ($p = 0.000$) (Fig. 5), independent of sex and HDM sensitization.

3.4. Effects of sex: female versus male groups

Independent of CAPs exposure and HDM sensitization, female mice presented an increase in BALF IL-5 ($p = 0.033$) and IL-17 levels ($p = 0.049$) (Fig. 5, C and D) and a reduction in BALF IL-2 ($p = 0.001$), IL-4 ($p = 0.000$), and TNF- α levels ($p = 0.003$) (Fig. 5, A, B and E).

3.5. Lung hyperresponsiveness

There were no effects observed for the Ers in the dose-response curve (see Table S4, online supplement).

All (♀ and ♂). Rrs was increased in the dose-response curve at baseline ($p = 0.026$), and with the administration of saline ($p = 0.018$), Mch 3 mg/mL ($p = 0.004$), Mch 30 mg/mL ($p = 0.010$), and Mch 300 mg/mL ($p = 0.002$) (Fig. 2, A), and the maximum MCh response ($p = 0.008$) (Fig. 2, D) was found in the HDM group compared with the FA group. Rrs was also significantly increased in the dose-response curve with the administration of Mch 300 mg/mL ($p = 0.026$) in the HDM group compared with the CAPs+HDM group (Fig. 2, A).

Females (♀). Rrs was increased in the dose-response curve at baseline ($p = 0.024$), and with the administration of saline ($p = 0.033$), Mch 3 mg/mL ($p = 0.007$), and Mch 30 mg/mL ($p = 0.021$) (Fig. 2, B) in the HDM group compared with the FA group. The maximum MCh response of Ers was significantly increased in the CAPs+HDM group compared

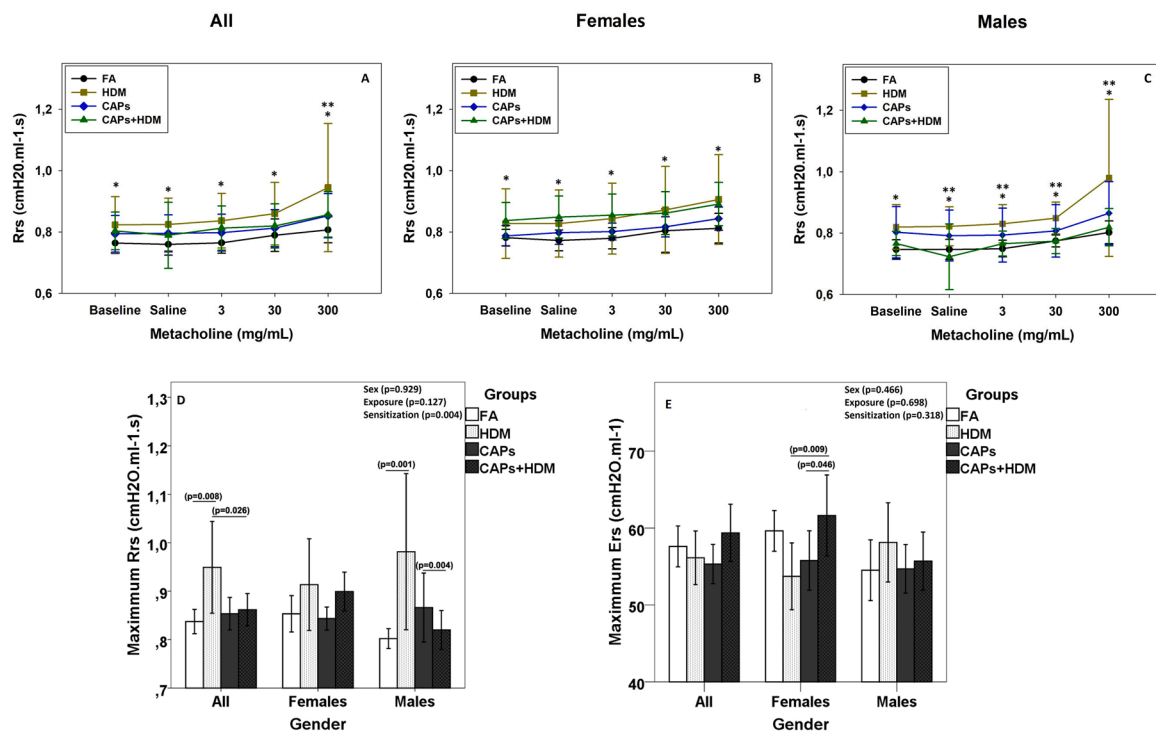


Fig. 2. Mean ($\pm$ SD) values of the dose-response curve to methacholine and maximum response to lung mechanics. **A, B and C.** Rrs, Resistance; **D.** Maximum Rrs; **E.** Maximum Ers, Elastance. FA, filtered air; HDM, house dust mites; CAPs, concentrated ambient particles; CAPs+HDM, CAP exposure + HDM sensitized. Dose-response curve effects at the Rrs: sex ($p = 0.394$), exposure ($p = 0.150$), and sensitization ($p = 0.001$). *HDM $\neq$ control [baseline (A, $p = 0.026$; B, $p = 0.024$; C, $p = 0.014$), saline (A, $p = 0.018$; B, $p = 0.033$; C, $p = 0.031$), 3 MCh (A, $p = 0.004$; B, $p = 0.007$; C, $p = 0.008$), 30 MCh (A, $p = 0.010$; B, $p = 0.021$; C, $p = 0.029$), and 300 MCh (A and C $p = 0.002$); **HDM $\neq$ CAPs+HDM [saline (C, $p = 0.005$), 3 MCh (C, $p = 0.033$), 30 MCh (C, $p = 0.029$), and 300 MCh (A, $p = 0.026$; C, $p = 0.004$)].

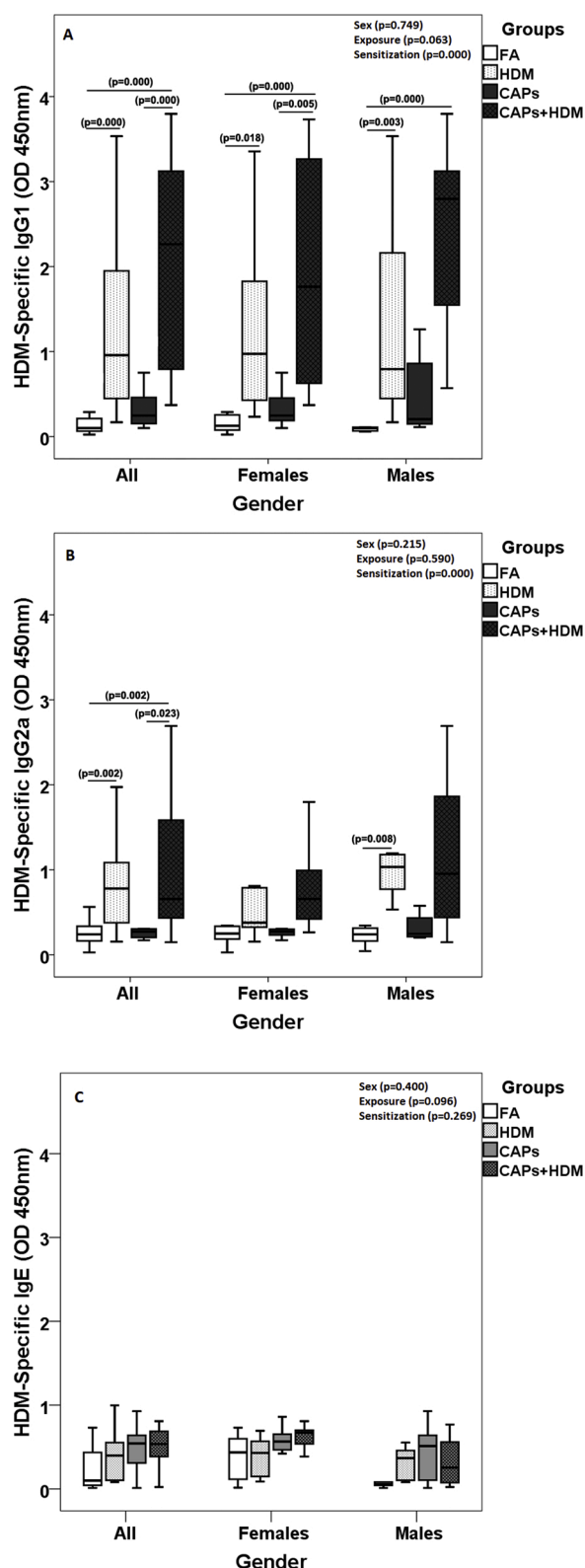


Fig. 3. IgG1 and IgG2a serum levels. **A.** IgG1, Immunoglobulin 1; **B.** IgG2a, Immunoglobulin 2a. FA, filtered air; HDM, house dust mites; CAPs, concentrated ambient particles; CAPs+HDM, CAP exposure + HDM sensitized. Data are presented as the median (IQR).

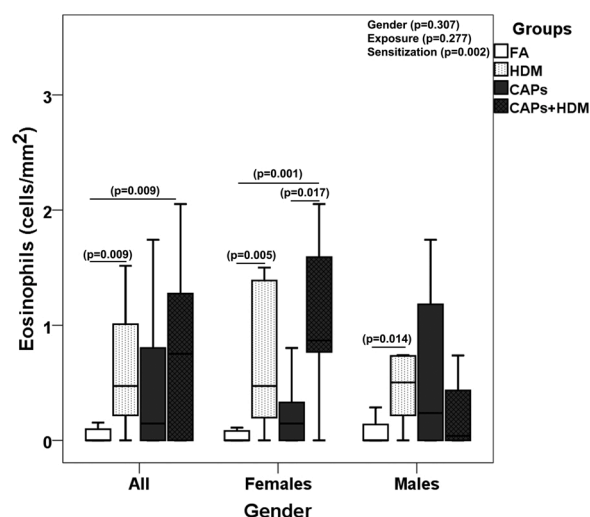


Fig. 4. Peribronchiolar eosinophils. FA, filtered air; HDM, house dust mites; CAPs, concentrated ambient particles; CAPs+HDM, CAP exposure + HDM sensitized. Data are presented as the median (IQR).

with the HDM ($p = 0.009$) and CAPs ($p = 0.046$) groups (Fig. 2, E).

Males (δ). Rrs was increased in the dose-response curve at baseline ($p = 0.014$), and with the administration of saline ($p = 0.031$), Mch 3 mg/mL ($p = 0.008$), Mch 30 mg/mL ($p = 0.029$), and Mch 300 mg/mL ($p = 0.002$) (Fig. 2, C), and the maximum MCh response ($p = 0.001$) (Fig. 2, D) was in the HDM group compared with the FA group. Rrs was also significantly increased in the dose-response curve with the administration of saline ($p = 0.005$), Mch 3 mg/mL ($p = 0.033$), Mch 30 mg/mL ($p = 0.029$), and Mch 300 mg/mL ($p = 0.004$) (Fig. 2, C), and the maximum MCh response ($p = 0.004$) (Fig. 3, D) was in the HDM group compared with the CAPs+HDM group.

3.6. Immunoglobulins (IgGs)

All (φ and δ). The HDM and CAPs+HDM groups showed increased levels of serum IgG1 ($p = 0.000$) and IgG2a ($p = 0.002$) compared to the FA group (Fig. 3, A and B). Serum IgG1 ($p = 0.000$) and IgG2a ($p = 0.023$) were also significantly increased in the CAPs+HDM group compared to the CAPs group (Fig. 3, A and B).

Females (φ). The HDM and CAPs+HDM groups showed increased levels of serum IgG1 ($p = 0.018$ and $p = 0.000$, respectively) compared to the FA group (Fig. 3, A). Serum IgG1 ($p = 0.005$) was also significantly increased in the CAPs+HDM group compared to the CAPs group (Fig. 3, A).

Males (δ). The HDM and CAPs+HDM groups showed increased levels of serum IgG1 ($p = 0.003$; $p = 0.000$) compared to the FA group (Fig. 3, A). Serum IgG2a ($p = 0.008$) increased in the HDM group compared to the FA group (Fig. 3, B).

There was no difference in the levels of serum IgE among groups (Fig. 3, C).

3.7. Inflammatory cells

All (φ and δ). The HDM and CAPs+HDM groups presented an increased density of eosinophils in the peribronchiolar region ($p = 0.009$) compared to the FA group (Fig. 4). The levels of BALF IL-2 ($p = 0.015$), IL-5 ($p = 0.000$), IL-17 ($p = 0.002$), and TSLP ($p = 0.041$) decreased in the HDM group compared to the FA group (Fig. 5, A, C, D, and F). The BALF IL-2 ($p = 0.005$), IL-4 ($p = 0.000$), and TNF- α ($p = 0.014$) levels were increased in the HDM group compared to the CAPs+HDM group (Fig. 5, A, B, and E). The CAPs+HDM group presented increased BALF IL-5 levels ($p = 0.041$) compared to the CAPs group (Fig. 5, C).

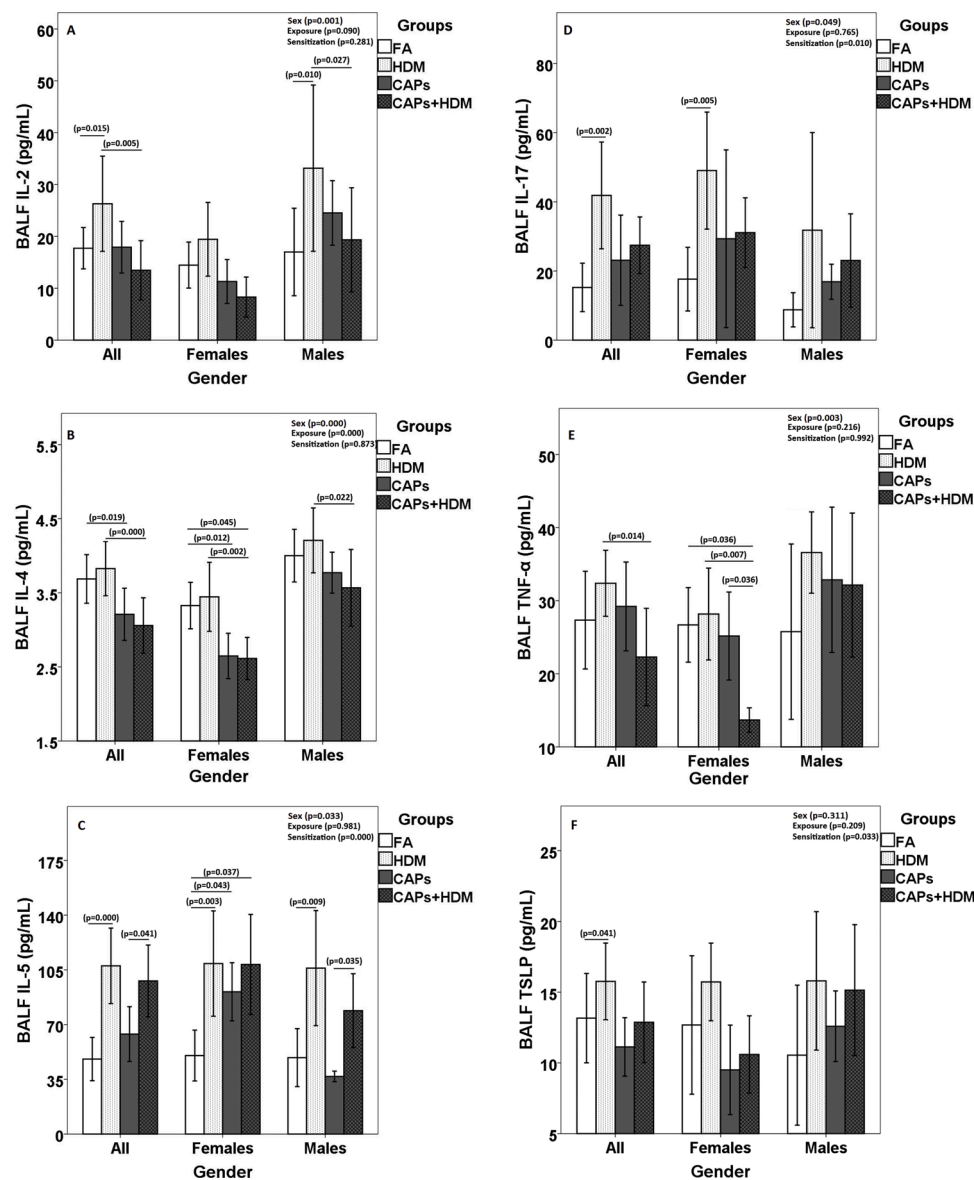


Fig. 5. Cytokine levels in BALF. FA, filtered air; HDM, house dust mites; CAPs, concentrated ambient particles; CAPs+HDM, CAP exposure + HDM sensitized. **A.** IL-2, Interleukin-2; **B.** IL-4, Interleukin-4; **C.** IL-5, Interleukin-5; **D.** IL-17, Interleukin-17; **E.** TNF- α , Tumour necrosis factor alpha; **F.** TSLP, Thymic stromal lymphoprotein. Data are presented as the mean ($\pm$ SD).

Females (♀). The peribronchiolar eosinophil density was higher in the HDM ($p = 0.005$) and CAPs+HDM ($p = 0.001$) groups than in the FA group. The peribronchiolar eosinophil density ($p = 0.001$) also increased in the CAPs+HDM group compared with the CAPs group (Fig. 4). The BALF results were as follows: (1) IL-4 levels were reduced in the CAPs+HDM group compared to the FA ($p = 0.045$) and HDM ($p = 0.002$) groups. The IL-4 levels ($p = 0.012$) were reduced in the CAPs group compared to the FA group (Fig. 5, B); (2) IL-5 levels were higher in the HDM ($p = 0.003$), CAPs ($p = 0.043$) and CAPs+HDM ($p = 0.037$) groups when compared to the FA group (Fig. 5, C); (3) IL-17 levels were increased in the HDM ($p = 0.005$) compared to the FA group (Fig. 5, D); and (4) TNF- α levels were reduced in the CAPs+HDM group when compared to the FA ($p = 0.003$), HDM ($p = 0.007$) and CAPs ($p = 0.036$) groups (Fig. 5, E).

Males (♂). There was an increase in the peribronchiolar eosinophil density ($p = 0.014$) in the HDM group compared to the FA group (Fig. 4) and in the alveolar tissue macrophage density ($p = 0.033$) in the CAPs+HDM group compared to the HDM group (Table 3). The BALF interleukin results were as follows: (1) IL-2 levels were higher in the

Table 3

Descriptive statistics of macrophage density in the lung parenchyma for all experimental groups (FA, HDM, CAPs, and CAPs+HDM). The results are expressed as the mean $\pm$ standard deviation (SD).

Sex	Group	N	Macrophages (cells/mm ²)		
All (♀ and ♂)	FA	18	47.06	$\pm$	9.44
	HDM	22	48.80	$\pm$	16.04
	CAPs	18	51.94	$\pm$	18.77
	CAPs+HDM	21	55.16	$\pm$	26.30
Females (♀)	FA	9	48.24	$\pm$	7.92
	HDM	10	50.86	$\pm$	17.26
	CAPs	11	51.87	$\pm$	15.43
	CAPs+HDM	13	48.69	$\pm$	23.36
Males (♂)	FA	9	45.88	$\pm$	11.11
	HDM	12	47.09	$\pm$	15.50
	CAPs	7	52.05	$\pm$	24.53
	CAPs+HDM	8	65.66	$\pm$	28.94*

FA, filtered air; HDM, house dust mites; CAPs, concentrated ambient particles; CAPs+HDM, CAP exposure + HDM sensitized. *HDM $\neq$ CAPs+HDM (δ , $p = 0.033$).

HDM group when compared with the FA ($p = 0.010$) and CAPs+HDM ($p = 0.027$) groups (Fig. 5, A); (2) IL-4 levels ($p = 0.022$) were reduced in the CAPs+HDM group when compared to the HDM group (Fig. 5, B); and (3) IL-5 levels were higher in the HDM group ($p = 0.020$) when compared to the FA group and in the CAPs+HDM group ($p = 0.035$) when compared to the CAPs group (Fig. 5, C). There were no differences in BALF cellularity among the groups (see Table S5, online supplement).

3.8. Methylation analysis

Methylation-specific PCR and direct DNA sequencing were used to evaluate the methylation status of the IL-4 promoter region. The methylation status of the IL-4 promoter region was successfully determined in 31 samples [FA ($n = 10$), HDM (6), CAPs ($n = 9$), and CAPs+HDM ($n = 6$)]. Representative results of the DNA methylation analysis are shown in Supplementary Figure S1. A total of 18 samples showed hemi-methylation of one of the analysable CpGs. However, the methylation status of the IL-4 promoter region was not significantly different between the animals exposed to CAPs or CAPs and HDM sensitization compared with the control group. No associations were found between the methylation status and the level of IL-4 protein expression (see Table S6, online supplement).

4. Discussion

In this study, we investigated whether intrauterine exposure to ambient levels of urban, vehicular-derived PM_{2.5} would be associated with increased lung allergic inflammation in juvenile (21d) sensitized mice. Our data showed that such exposure did not worsen lung hyper-responsiveness or allergic inflammation but altered the lung immune profile of young mice, which differed between the sexes. There was a decrease in the lung IL-4 (males and females), IL-2 (males), and TNF- α (females) cytokines, with increased macrophagic inflammation and hyporesponsiveness in males.

Our HDM model was capable of triggering airway hyper-responsiveness and increasing IgG1, BALF IL-5, and perivascular eosinophilia, confirming its adequacy to study allergic inflammation. There was a further increase in IL-17 levels in the females and IgG2a and BALF IL-2 in the males. On the other hand, CAPs exposure alone reduced BALF IL-4 in both sexes and increased IL-5 in female mice only. Our data confirm that the pulmonary inflammatory response to insults differs between the sexes in mice, which may lead to different cytokine profiles (Wang et al., 2020b; Yoshizaki et al., 2017). Female mice seem to be more susceptible to the development of allergic airway inflammation than male mice (Melgert et al., 2005).

Epidemiological data indicate that intrauterine exposure to PM_{2.5} is related to an increased risk of asthma in children, which is due to mechanisms such as altered lung structure, epigenetic changes, and immune dysregulation as reviewed by B. Wang et al. (2020a). Here, we have shown that juvenile sensitized mice exposed to ambient levels of intrauterine PM_{2.5} did not present signs of worsened allergic inflammation or airway hyperresponsiveness. In contrast, the levels of some lung (T_H2) cytokines were decreased, such as IL-4 and TNF- α in females and IL-2 in male mice. Males presented airway hyporesponsiveness with more lung tissue macrophages. Our data reinforce the results of previous studies showing the existence of sexual dimorphism in relation to lung function responses in different insults, such as pollutant exposure and viral infections, with males being hyporesponsive (Larcombe et al., 2011; Yoshizaki et al., 2017).

Previous experimental studies addressing the relation between intrauterine exposure and the development of allergic inflammation have shown contrasting results. Mice exposed to UFP in utero and challenged with HDM beginning at 3 days of age did not develop a robust airway inflammatory response compared with HDM-sensitized mice, suggesting mechanisms of immunosuppression and host defence response to oxidative stress induced by UFP (Rychlik et al., 2019). Intrauterine

exposure to low concentrations of formaldehyde suppressed the development of allergic lung inflammation with OVA beginning at 30 days of age by mechanisms mediated by reduced anaphylactic antibody synthesis and IL-6 and TNF- α secretion (Maiellaro et al., 2014). On the other hand, Fu et al. (2020) showed that prenatal ozone exposure exacerbated the severity of OVA-induced asthma in pups, which might be directly caused by the increased NK (natural killer) cells in offspring. Similar results were found in studies using diesel exhaust (Manners et al., 2014) or ROFA (Hamada et al., 2007) in allergically sensitized pups.

It is possible that ambient levels of exposure, such as in this study and the studies by Rychlik et al. (2019) (low-dose UFPs) and Maiellaro et al. (2014) (low-dose formaldehyde), were not capable of inducing T_H2 skewing in the offspring. Rychlik et al. (2019) further suggested that low PM exposure may cause immunosuppression, with decreased T_H2/T_H17 cytokines and increased IL-10 levels. Our data showed a decrease in TNF- α levels in female mice, which may have implications in the response to infectious agents (Maiellaro et al., 2014). Indeed, some studies have shown an association between intrauterine exposure to PM_{2.5} and higher rates of respiratory infection in children (Jedrychowski et al., 2013; S. Yang et al., 2020).

Another possible reason for the lack of increased allergic responses in our model was the age of initial sensitization. The T_H2 milieu prevails in the postnatal lung (days 10–14), with the influx of eosinophils in the lung as demonstrated by Loffredo et al. (2020). It is possible that models beginning with early postnatal sensitization more easily perpetuate allergic inflammation (Fu et al., 2020; Hamada et al., 2007).

Macrophage polarization has been associated with the progression of asthma because of its critical role in host defence and homeostasis (Saradna et al., 2018). Robbe et al. (2015) demonstrated that mice sensitized with farm dust extract showed polarization towards M1 macrophages and that HDM sensitization generated M2 polarization. We did not analyse macrophage subtyping in this study, but the decreased BALF IL-4 and IL-2 associated with an increased density of tissue macrophages in male mice could be related to a different macrophage phenotype switch in relation to allergic inflammation associated with PM_{2.5} exposure.

The metallic and organic composition of the PM_{2.5} to which animals were exposed induced DNA damage in the lungs of mice (de Oliveira Alves et al., 2020). Epigenetic events, especially DNA methylation changes, have been associated with an increased risk of developing allergic phenotypes and asthma (DeVries and Vercelli, 2016). Altered DNA methylation status of the promoter region of different genes has been associated with PM exposure in different life stages (Ferrari et al., 2019). A recent study performing epigenome-wide meta-analysis provides evidence of new-born methylation differences in several genes with relevance for airway disease in relation to prenatal particulate air pollution exposure (Gruzdeva et al., 2019). Here, we were unable to detect statistically significant differences in the status of DNA methylation of the IL-4 promoter region in our animal model in relation to exposure to CAPs or CAPs+HDM.

Our study has some limitations, especially related to the number of animals per group and the number of CpGs evaluated in the epigenetic analyses, and future studies are required to better understand the epigenetic mechanisms associated with PM and asthma development. In this study, the mice have received an average of 600 $\mu\text{g}/\text{m}^3$ PM_{2.5} during 1 h corresponding to a daily average of 25 $\mu\text{g}/\text{m}^3$, which is the maximum daily PM_{2.5} recommended by the WHO (WHO. World Health Organization, 2005). In fact, in 2019 the maximal daily concentrations of PM_{2.5} in Sao Paulo (mainly of vehicular origin) were much higher than this value ($\sim 50 \mu\text{g}/\text{m}^3$) [CETESB and Companhia Ambiental do Estado de São Paulo, 2020]. We implemented an experimental setup exposing animals to CAPs for a specified time per day, rather than exposing for 24 h to simulate ambient conditions (Maud et al., 2008), to avoid environmental fluctuations. We established an exposure setup with more constancy but higher delivered particle doses. Such exposure to higher pollutant levels over a short period of time may lead to

physiological changes different from those caused by a long-time low doses exposure (de Barros et al., 2018), which is an important limitation of this study.

We have chosen the dose of 100 µg HDM based on several reports that were able to induce allergic inflammation in different types of exposures (Ferrini et al., 2017; Timmerman et al., 2019; van Rijt et al., 2012; Vieira et al., 2011; Woo et al., 2018). Since our main goal was to study the influence of intrauterine PM_{2.5} exposure in the development of allergic inflammation later in life, we used a 3-week duration model, when allergic features will be established (Woo et al., 2018).

5. Conclusion

In summary, intrauterine exposure to ambient PM_{2.5} did not increase allergic pulmonary susceptibility but altered the immune profile of juvenile sensitized mice, with increased macrophagic inflammation and a decrease in cytokines, and the profile differed by sex.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.tox.2021.152970>.

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Effects of intrauterine exposure to concentrated ambient particles on allergic sensitization in juvenile mice

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[Supplementary material](#)

2. Methods

Table S1. Descriptive statistics of pregnant weight for experimental groups (FA and CAPs). The results are expressed as the mean, minimum (Min) and maximum (Max).

Weight (grams)	FA (n=10)		CAPs (n=10)	
	Mean	Min - Max	Mean	Min - Max
DG 1	20	18 - 22	19	18 - 22
DG 6	23	19 - 25	22	20 - 26
DG 12	26	24 - 28	26	20 - 31
DG 18	31	26 - 36	32	25 - 38

FA, filtered air; **CAPs**, concentrated ambient particles; **DG**, day gestational. During the gestational period, four weight measurements were taken before litter birth.

Table S2. Descriptive statistics of offspring body weight for all experimental groups (FA, HDM, CAPs, and CAPs+HDM). The results are expressed as the mean, minimum (Min) and maximum (Max).

Weight (grams)	Females (♀)							
	FA (n=11)		HDM (n=12)		CAPs (n=11)		CAPs+HDM (n=13)	
	Mean	Min - Max	Mean	Min - Max	Mean	Min - Max	Mean	Min - Max
PND 21	8	5 - 11	7	4 - 11	7	5 - 11	8	5 - 11
PND 24	9	5 - 13	10	6 - 13	9	7 - 11	9	7 - 12
PND 27	11	5 - 15	12	8 - 16	10	8 - 13	11	7 - 15
PND 31	14	5 - 18	13	10 - 17	13	11 - 16	14	10 - 16
PND 35	15	5 - 19	16	11 - 20	15	12 - 17	15	12 - 18
PND 39	16	5 - 19	16	11 - 18	16	14 - 19	17	13 - 19
PND 43	17	5 - 20	17	13 - 19	16	14 - 19	17	13 - 20

Weight (grams)	Males (♂)							
	FA (n=12)		HDM (n=14)		CAPs (n=8)		CAPs+HDM (n=9)	
	Mean	Min - Max	Mean	Min - Max	Mean	Min - Max	Mean	Min - Max
PND 21	8	6 - 10	8	6 - 12	9	7 - 11	8	5 - 11
PND 24	10	8 - 14	10	8 - 14	10	8 - 13	10	8 - 12
PND 27	13	11 - 16	13	10 - 16	14	11 - 15	13	10 - 15
PND 31	16	12 - 21	16	12 - 19	17	15 - 19	17	14 - 19
PND 35	18	15 - 23	18	13 - 20	19	18 - 20	18	15 - 21
PND 39	19	16 - 23	19	16 - 20	20	19 - 22	19	16 - 21
PND 43	20	17 - 23	20	18 - 22	21	18 - 24	21	19 - 24

FA, filtered air; **HDM**, house dust mites; **CAPs**, concentrated ambient particles; **CAPs+HDM**, CAP exposure + HDM sensitized; **PND**, postnatal day. After lactation period, weight was recorded twice a week, totalling seven measurements before euthanasia.

3. Results

Non-significant results

Table S3. Effects of PM_{2.5} concentrations. Values are expressed as the mean $\pm$ SD.

	FA	CAPs
Fertility	100%	80%
Gestational length (days)	19.5 $\pm$ 2.2	18.8 $\pm$ 1.5
Pregnant weight (grams)	31.0 $\pm$ 3.0	32.4 $\pm$ 4.9
Litter size (animals number)	(m: 22, f: 21)	(m:21, f: 26)
Male:female ratio	1.33 $\pm$ 0.88	0.96 $\pm$ 0.69

CAPs, concentrated ambient particles; **PM_{2.5}**, particulate matter less than 2.5 micrometres; **m**, male; **f**, female

Table S4. Descriptive statistics of the dose-response curve to methacholine (MCh) of Ers for all experimental groups (FA, HDM, CAPs, and CAPs+HDM). The results are expressed as the mean $\pm$ standard deviation (SD).

Sex	Group	N	Ers (cmH ₂ O.ml ⁻¹)				
			Baseline	Saline	3 Mch	30 Mch	300 Mch
All (♀ and ♂)	FA	20	42.30 $\pm$ 3.79	49.50 $\pm$ 4.87	52.93 $\pm$ 4.70	55.49 $\pm$ 4.87	58.58 $\pm$ 5.76
	HDM	23	41.84 $\pm$ 6.37	46.65 $\pm$ 7.83	50.76 $\pm$ 8.94	54.39 $\pm$ 12.34	58.71 $\pm$ 16.39
	CAPs	19	42.92 $\pm$ 4.80	46.87 $\pm$ 5.02	50.39 $\pm$ 5.32	52.66 $\pm$ 5.52	55.32 $\pm$ 5.56
	CAPs+HDM	22	42.55 $\pm$ 5.01	51.16 $\pm$ 9.05	53.26 $\pm$ 6.99	55.61 $\pm$ 6.97	58.24 $\pm$ 7.33
Females (♀)	FA	11	44.00 $\pm$ 2.38	51.62 $\pm$ 3.45	55.18 $\pm$ 3.11	57.23 $\pm$ 3.68	59.63 $\pm$ 4.38
	HDM	11	40.45 $\pm$ 6.22	46.25 $\pm$ 6.58	50.83 $\pm$ 8.49	55.06 $\pm$ 15.37	60.05 $\pm$ 21.98
	CAPs	11	43.88 $\pm$ 5.36	47.63 $\pm$ 5.14	51.12 $\pm$ 5.41	53.29 $\pm$ 5.85	55.78 $\pm$ 6.41
	CAPs+HDM	13	43.05 $\pm$ 5.26	53.31 $\pm$ 10.46	54.81 $\pm$ 7.83	57.14 $\pm$ 7.69	59.81 $\pm$ 8.11
Males (♂)	FA	9	40.67 $\pm$ 4.50	47.06 $\pm$ 5.09	50.61 $\pm$ 5.14	53.97 $\pm$ 5.77	57.92 $\pm$ 7.24
	HDM	12	43.11 $\pm$ 6.50	47.01 $\pm$ 9.11	50.70 $\pm$ 9.71	53.77 $\pm$ 9.44	57.47 $\pm$ 9.72
	CAPs	8	41.61 $\pm$ 3.84	45.81 $\pm$ 4.99	49.39 $\pm$ 5.37	51.80 $\pm$ 5.29	54.70 $\pm$ 4.47
	CAPs+HDM	9	41.73 $\pm$ 4.81	47.67 $\pm$ 4.88	50.74 $\pm$ 4.76	53.13 $\pm$ 5.10	55.70 $\pm$ 5.35

FA, filtered air; HDM, house dust mites; CAPs, concentrated ambient particles; CAPs+HDM, CAP exposure + HDM sensitized; Ers, elastance; Mch, methacholine.

Table S5. Descriptive statistics of inflammatory cells in bronchoalveolar lavage fluid (BALF) for all experimental groups (FA, HDM, CAPs, and CAPs+HDM). The results are expressed as the median and interquartile range (IQR).

Sex	Group	N	Total Cells	Macrophages	Neutrophils	Lymphocytes	Eosinophils
All (♀ and ♂)	FA	23	5.10 2.90 - 6.00	3.45 1.60 - 4.79	0.00 0.00 - 0.02	1.22 0.91 - 2.02	0.00 0.00 - 0.00
	HDM	25	5.90 4.00 - 8.80	3.65 2.50 - 5.81	0.02 0.00 - 0.06	1.84 1.24 - 2.91	0.00 0.00 - 0.02
	CAPs	21	3.90 2.60 - 5.60	1.37 0.78 - 4.03	0.00 0.00 - 0.01	1.47 0.75 - 2.48	0.00 0.00 - 0.00
	CAPs+HDM	22	5.30 3.55 - 7.90	2.83 0.75 - 6.96	0.00 0.00 - 0.04	1.24 0.74 - 2.79	0.02 0.00 - 0.04
Females (♀)	FA	11	5.30 4.90 - 7.50	4.19 1.61 - 5.21	0.00 0.00 - 0.01	1.22 0.94 - 2.38	0.00 0.00 - 0.00
	HDM	12	5.65 3.55 - 9.20	3.69 2.42 - 6.98	0.02 0.01 - 0.07	1.55 1.07 - 2.68	0.00 0.00 - 0.02
	CAPs	11	4.20 2.60 - 5.80	3.74 0.50 - 4.78	0.00 0.00 - 0.00	1.50 1.07 - 1.90	0.00 0.00 - 0.00
	CAPs+HDM	13	5.70 4.10 - 8.40	4.91 0.60 - 6.94	0.00 0.00 - 0.05	1.21 0.78 - 3.54	0.01 0.00 - 0.02
Males (♂)	FA	12	4.25 2.85 - 5.85	2.77 1.56 - 3.71	0.01 0.00 - 0.03	1.21 0.72 - 1.75	0.00 0.00 - 0.02
	HDM	13	6.40 4.70 - 7.00	3.65 2.50 - 4.75	0.00 0.00 - 0.04	2.52 1.56 - 3.24	0.00 0.00 - 0.02
	CAPs	10	3.80 1.90 - 5.10	1.25 0.97 - 2.85	0.01 0.00 - 0.02	1.24 0.55 - 3.00	0.00 0.00 - 0.01
	CAPs+HDM	9	3.80 3.00 - 7.40	1.22 1.03 - 6.98	0.00 0.00 - 0.03	1.94 0.30 - 2.44	0.03 0.00 - 0.05

FA, filtered air; **HDM**, house dust mites; **CAPs**, concentrated ambient particles; **CAPs+HDM**, CAP exposure + HDM sensitized.

Table S6. IL-4 protein expression profile in each sample group.

Sex	Group	N	CpG Partially Methylated			N	CpG Methylated		
All (♀ and ♂)	FA	4	3.33	±	0.51	5	4.01	±	0.80
	HDM	3	3.18	±	0.05	3	3.37	±	1.09
	CAPs	7	2.61	±	0.65	2	3.32	±	0.06
	CAPs+HDM	4	2.65	±	0.36	2	3.07	±	0.35

FA, filtered air; **HDM**, house dust mites; **CAPs**, concentrated ambient particles; **CAPs+HDM**, CAP exposure + HDM sensitized.

The T-test or ANOVA was performed for group comparisons.

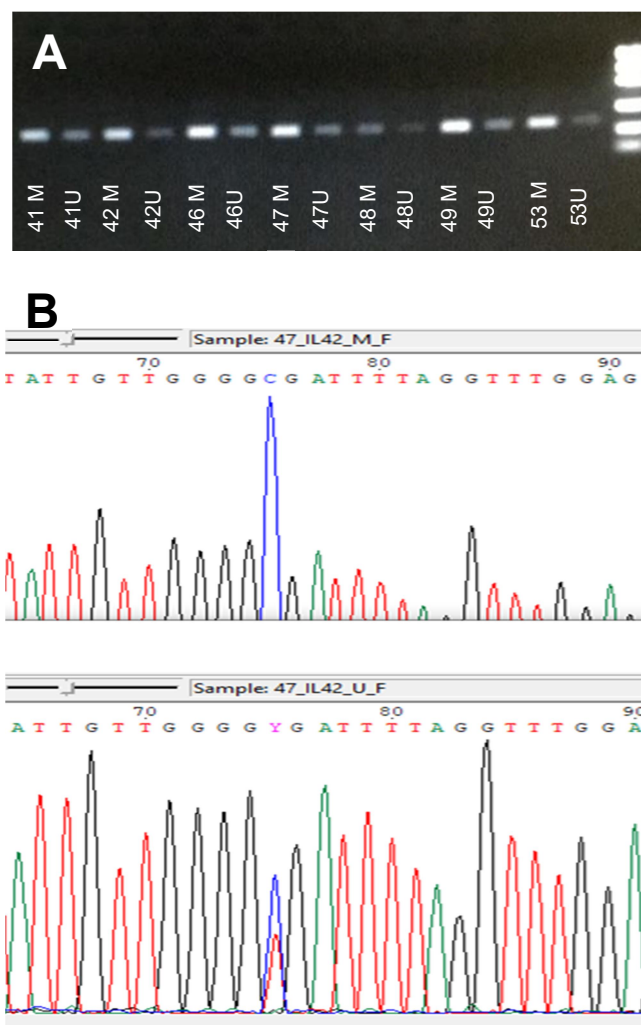


Figure S1. DNA methylation analysis of mouse IL-4. **A.** Representative MSP-PCR gel electrophoresis of bisulfite-converted genomic DNA from mouse lungs. **B.** Sequencing electropherogram of hemi-methylated CpG from IL-4. **M.** Amplicon for methylated DNA; **U.** Amplicon for unmethylated DNA.