



Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Inflammatory and functional responses after (bio)diesel exhaust exposure in allergic sensitized mice. A comparison between diesel and biodiesel[☆]

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ARTICLE INFO

Article history:

Received 13 November 2018

Received in revised form

23 April 2019

Accepted 21 June 2019

Available online 5 July 2019

ABSTRACT

Many cities fail to meet air quality standards, which results in increased risk for pulmonary disorders, including asthma. Human and experimental studies have shown that diesel exhaust (DE) particles are associated with worsening of allergic asthma. Biodiesel (BD), a cleaner fuel from renewable sources, was introduced in the eighties. Because of the reduction in particulate matter (PM) emissions, BD was expected to cause fewer adverse pulmonary effects. However, only limited data on the effect of BD emissions in asthma are available.

Objective: Determine whether BD exhaust exposure in allergic sensitized mice leads to different effects on inflammatory and functional responses compared to DE exposure.

Methods: Balb/C mice were orotracheally sensitized with House Dust Mite (HDM) or a saline solution with 3 weekly instillations. From day 9 until day 17 after sensitization, they were exposed daily to filtered air (FA), DE and BD exhaust (concentration: 600 µg/m³ PM_{2.5}). Lung function, bronchoalveolar lavage fluid (BALF) cell counts, cytokine levels (IL-2, IL-4, IL-5, IL-17, TNF-α, TSLP) in the BALF, peribronchiolar eosinophils and parenchymal macrophages were measured.

Results: HDM-sensitized animals presented increased lung elastance ($p = 0.046$), IgG1 serum levels ($p = 0.029$), peribronchiolar eosinophils ($p = 0.028$), BALF levels of total cells ($p = 0.020$), eosinophils ($p = 0.028$), IL-5 levels ($p = 0.002$) and TSLP levels ($p = 0.046$) in BALF. DE exposure alone increased lung

[☆] This Paper has been recommended for acceptance by Wen Chen.

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elastance ($p = 0.000$) and BALF IL-4 levels ($p = 0.045$), whereas BD exposure alone increased BALF TSLP levels ($p = 0.004$). BD exposure did not influence any parameters after HDM challenge, while DE exposed animals presented increased BALF levels of total cells ($p = 0.019$), lymphocytes ($p = 0.000$), neutrophils ($p = 0.040$), macrophages ($p = 0.034$), BALF IL-4 levels ($p = 0.028$), and macrophagic inflammation in the lung tissue ($p = 0.037$), as well as decreased IgG1 ($p = 0.046$) and IgG2 ($p = 0.043$) levels when compared to the HDM group.

Conclusion: The results indicate more adverse pulmonary effects of DE compared to BD exposure in allergic sensitized animals.

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Abbreviations

AHR	Airway hyperresponsiveness
AUC	Area under the curve
BALF	Bronchoalveolar lavage
BD	Biodiesel
BSA	Bovine serum albumin
DE	Diesel exhaust
DEP	Diesel exhaust particles
Ers	Elastance/elastic resistance
FA	Filtered air
HDM	House dust mite
IL	Interleukin
Mch	Methacholine

NO	Nitric oxide
NO ₂	Nitrogen dioxide
NO _x	Nitrogen oxides
OVA	Ovalbumin
PBS	Phosphate buffered saline
PM	Particulate matter
PM _{2.5}	Particulate matter minor that 2.5 µm
Rrs	Pulmonary resistance
SO ₂	Sulfur dioxide
SOL	Solid spherical carbon particles
Th2	T-helper type 2 cells
Th17	T-helper type 17 cells
TNF-α	Tumor necrosis factor-alpha
TSLP	Thymic Stromal Lymphopoietin

1. Introduction

Air pollution has a known adverse impact on cardiopulmonary human health (International Energy Agency, 2016). Diesel-fueled heavy vehicles are the largest emitters of particulate matter (PM), nitrogen oxides (NO_x), and sulfur dioxide (SO₂), which have been consistently associated with worsening of pulmonary diseases (Akopian et al., 2016; IEA, 2016). Diesel exhaust (DE) is composed of gaseous and aerosol phases with different toxic substances. The solid phase contains spherical carbon particles with an aerodynamic diameter of 10–80 nm that become coated with toxic organic and inorganic components. When inhaled, these particles can be deposited in the lungs, being consistently associated with adverse health outcomes (Gangwar et al., 2011).

The increase in asthma prevalence in cities has been attributed to accelerated urbanization, high levels of vehicle emissions, and a western lifestyle (Carlsten and Georas, 2014; Li et al., 2011). Exposure to traffic-derived air pollution can exacerbate disease in asthmatics or predispose children to allergic sensitization (Li et al., 2011). Asthmatics are potentially more susceptible to the effects of diesel than healthy individuals (Brandt et al., 2015). Diesel exhaust particles (DEPs) stimulate T-helper type 2 cells (Th2) and the secretion of T2 cytokines such as interleukin (IL)-4, IL-5, IL-9, and IL-13 (Alexis and Carlsten, 2014). The organic chemical substances present in DEPs might also aggravate asthma (Takano and Inoue, 2017).

Different animal models have shown that DEPs are associated with worsening of allergic asthma (Acciani et al., 2013; Brandt et al., 2015, 2013; Tanaka et al., 2013). Repeated exposure to low-dose DE (100 µg/m³ of PM_{2.5}) in murine asthma models after (not during) allergic sensitization with ovalbumin (OVA) resulted in increased airway hyperresponsiveness (AHR) and gene expression of pro-asthmatic chemokines and cytokines. These effects were most

abundant after short-term exposure (1 day) and faded after prolonged DE exposure (8 weeks) (Matsumoto et al., 2006). Another study demonstrated that instilled intratracheal DEPs and House Dust Mite (HDM) co-exposure resulted in increased accumulation of HDM-specific memory Th2 and Th17 cells in the lungs, potentiating secondary recall responses and promoting the development of allergic asthma (Brandt et al., 2015).

Currently, the development of new types of fuels to replace petrol derivatives is a key challenge for biotechnological research. In recent years, biodiesel (BD) has received considerable attention, as it is derived from different renewable vegetal or animal sources. BD is composed of alkyl esters of long-chained fatty acids derived from vegetable oils (palm, soybean, rapeseed, and castor) or animal fats (ANP, 2012). When combusted, BD reduces exhaust emissions of PM, carbon monoxide, carbon dioxide, and hydrocarbons at significant proportions (Cheng et al., 2017; Graver et al., 2016; Silitonga et al., 2017; Tsai et al., 2014), suggesting that BD would offer an attractive solution to reduce greenhouse gases and potentially reduce health risks (Lankoff et al., 2017; Martin et al., 2017).

There are still few studies on the respiratory health effects caused by BD fuel emissions. Shvedova et al. (2013) compared lung toxicity, inflammatory response, and oxidative stress production after a 4-week exposure to DE and BD exposure and showed an increased level in all three parameters for the animals exposed to BD compared to DE exposition. Additionally, Yanamala et al. (2013) showed a higher inflammatory response by detecting an increased number of chemokines/cytokines in lung tissue after BD exposure compared to acute DE exposure.

There is scarce information on the effects of exposure to BD exhaust in asthma, especially on the effects of biodiesel exposure on asthma exacerbation. Gavett et al. (2015) showed that different concentrations of soy biodiesel exposure during allergic

sensitization in mice did not induce inflammatory responses, contrary to diesel exhaust exposure. In this study, we tested, in a model of acute allergic asthma, whether previously HDM-sensitized animals exposed to soy/tallow biodiesel would respond differently than DE-exposed animals with respect to functional and inflammatory parameters. Our hypothesis was that differences in fuel composition could differently affect allergic responses in mice.

2. Material and methods

2.1. Ethics statement

This study was approved by the Ethical Committee of the School of Medicine of the University of São Paulo (n° 150/16).

2.2. Fuels and exposure system

We used São Paulo Metropolitan diesel (10 ppm of sulfur with a blend of 5% soy biodiesel) and 60% soybean + 40% tallow methyl esters biodiesel 100% - B100, obtained from the Laboratory of Quality Control of the Biodiesel Factory of Montes Claros (Minas Gerais, Brasil), Brazilian Petroleum Corporation — Petrobras. The physical properties of DE fuel were analyzed by the Laboratory for Lubricating and Combustible Oils of the National Service of Industrial Apprenticeship (São Paulo, Brasil), and the BD analysis was performed by the fuel provider (see [Tables S1 and S2](#), online supplement). Polycyclic Aromatic Hydrocarbon (PAH) characterization in both fuels was performed at the laboratory of the Technological Research Institute of São Paulo; detailed information on the methodology used is provided in the Supplementary Material (see [S3](#), online supplement). Protocol of the exposure to DE and BD exhaust ([Fig. 1](#)) was performed as previously described by [de Brito et al. \(2018\)](#).

2.3. Sampling, and PM_{2.5} characterization

The 1-h concentration of PM_{2.5} was determined gravimetrically using Harvard impactors (Air Diagnostics, Harrison, ME) at a flow rate of 10 L m⁻¹. Impactors were equipped with 37 mm polycarbonate filters with a 0.8- μ m pore size (Isopore Membrane Filters, polycarbonate, 0.8 μ m, 37 mm; Millipore). The elements sodium (Na), magnesium (Mg), aluminum (Al), sulfur (S), potassium (K), calcium (Ca), manganese (Mn), iron (Fe), copper (Cu), zinc (Zn), rubidium (Rb), barium (Ba), chromium (Cr), arsenic (As), vanadium (V), silicon (Si), and lead (Pb) were quantified using an energy dispersive X-ray fluorescence spectrometer (EDX700-HS, Shimadzu Corporation Analytical Instruments Division, Kyoto, Japan). This instrument uses a low-power Rh-target tube, with a voltage of 5–50 kV and a current of 1 to 1000 μ A. The characteristic X-ray radiation was detected with a Si (Li) detector. X-ray fluorescence emission spectra of elements were collected for 400 s from a 10-mm sample surface area under a vacuum atmosphere. The measured sample intensities were converted to element concentrations (ng.cm²) through calibration with the NIST 2783 (Air Particulate on Filter Media) standard reference materials (National Institute of Standards, Gaithersburg, MD, USA). Finally, carbon was used for mass balance ([Carvalho-Oliveira et al., 2015](#); [Mauad et al., 2008](#)). Ten filters were collected, and all analyses were performed in triplicate.

2.4. Animals

Adult male Balb/C mice (6–8 weeks of age) weighing ~25 g were obtained from the animal house at the School of Medicine of University of São Paulo. We used male mice to avoid inflammatory responses influenced by hormonal variations that occur in females ([Ligeiro de Oliveira et al., 2004](#); [Riffo-Vasquez et al., 2007](#); [Yoshizaki](#)

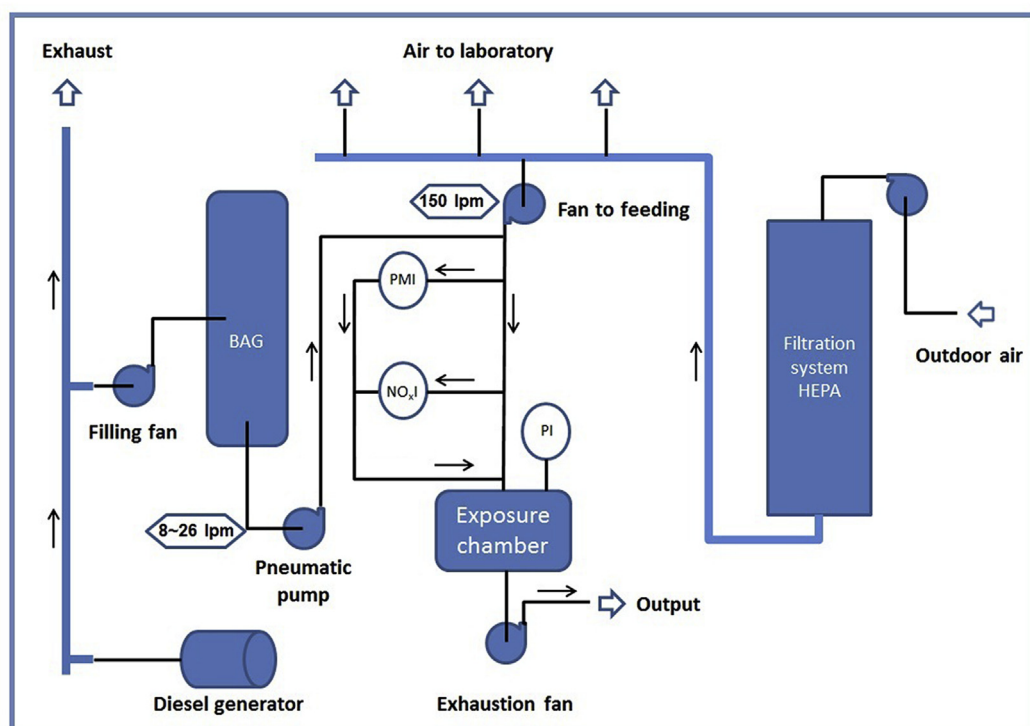


Fig. 1. Schematic drawing of the exhaust generator system used in the study. PM, particulate matter; NO_x, nitrogen oxides; PI, internal pressure; lpm, liter per minute. Design by Paulo Afonso de André.

et al., 2017). The animals were maintained at 22 °C – 23 °C with controlled humidity and a 12- to 12-h light cycle before exposure for acclimatization (3 days) and between exposure (Organisation for Economic Co-operation and Development (OECD, 2010). Food and water were available *ad libitum*. All animals received care in compliance with the Guide for the Care and Use of Laboratory Animals of the National Academies (The National Academies Press, 2010).

2.5. Study design

Six experimental groups with 11–12 animals each were defined as follows: (1) filtered air (FA): control; (2) DE: exposed to diesel exhaust; (3) BD: exposed to biodiesel exhaust; (4) House dust mite (HDM): exposed to FA and HDM-sensitized; (5) DE+HDM: exposed to DE and HDM-sensitized; and (6) BD+HDM: exposed to BD and HDM-sensitized (See Table S4 for the number of animals used in the different procedures, online supplement).

Sensitized animals received three oro-tracheal with 30 µL of phosphate-buffered saline (PBS) containing 100 µg of *Dermaphagoides pteronyssinus* (Greer Laboratories, Lenoir, NC, USA), the House Dust Mite (HDM), as previously described (Vieira et al., 2011). Control mice received oro-tracheal instillations of saline. The solutions were homogenized with a Vortex mixer (Phoenix AP-56, Brazil) during 30 s before use. The acute model consisted of a daily exposure of 1 h, from day 9 to day 17, to one of FA, 600 µg/m³ PM_{2.5} of DE, or 600 µg/m³ PM_{2.5} of BD corresponding to a daily average of 25 µg/m³, which is the maximum daily PM_{2.5} recommended by the World Health Organization (WHO, World Health Organization, 2005). On day 18, the day after the last exposure, the animals were sacrificed and lung function, blood and BALF samples, and lung tissue were collected (Fig. 2).

2.6. Lung function analysis

Animals were anesthetized with sodium pentobarbital (50 mg/kg i.p.), tracheostomized and connected to a small ventilator (Harvard 683; Harvard Apparatus Co., South Natick, MA, USA), which provided the animals with a respiratory frequency of 150 times/min with a volume of 10 mL/kg. To measure the airflow (V), a pneumatachograph (Fleish 4-0; OEM Medical, Richmond, VA, USA) and a pressure transducer (Honeywell 163PC01D36; Honeywell, Freeport, IL, USA) were connected to the trachea cannula. A different pressure transducer (Honeywell 142PC05D; Honeywell) was used to measure the tracheal pressure (Pt). Crescent concentrations (saline, 3, 30 and 300 mg/mL) of methacholine (Mch) generated by an ultrasonic nebulizer connected to an inlet valve of a Harvard 683 mice-ventilator were inhaled for 1 min each in intervals of 3 min. This system requires high doses of Mch because of the size of the circuit between the nebulizer and the trachea of the

mice (Arantes-Costa et al., 2008; Bortolozzo et al., 2018; Brüggemann et al., 2017; Santos et al., 2018). After every administration, Pt and V' measurements were taken 30 s, 1 min, 2 min and 3 min after the Mch challenge.

The data was collected and measurements were calculated by a 12-bit analog-to-digital converter (DT 01-EZ; Data Translation, Marlboro, MA). Using the parameters V' and Pt, respiratory system resistance (Rrs) and respiratory system elastance/elastic resistance (Ers) were calculated according the following equation: $P_{tr} = E_{rs} * V + R_{rs} * V'$. The highest dose, regardless of the time, was chosen to determine maximal respiratory response. We analyzed the area under the curve (AUC).

2.7. HDM-specific IgE, IgG1, IgG2a

After AHR test, until under anesthesia, blood samples were collected through the cava vein, the samples were centrifuged (1000 rpm, 4 °C, 20 min). Serum samples were stored at –70 °C until the assay was performed as previously described (Olivo et al., 2012). Finishing this procedure, mice were sacrificed by exsanguination.

For IgE, IgG1 and IgG2a, high binding microplate was covered with HDM (100 µg/mL per well) and incubated per 2 h in a humid chamber at 37 °C. After the incubation, the plaques were washed, blocked with PBS+10% bovine serum albumin (BSA) (200 µL/well) and incubated per 3 h. The plates were washed and were added samples serum (100 µL/well). The plates were stored overnight on a refrigerator. Next, the plates were washed and to develop the reaction, IgE-specific (1/8000), IgG1 (1/2000) and IgG2a (1/4000) detection biotinylated antibodies were added, followed by incubation per 2 h and washing. The developing solution containing the enzymatic conjugate of streptavidin-peroxidase, substrate, and chromogen were added. The color reaction was read in a 490 nm spectrophotometer (Brüggemann et al., 2017).

2.8. Bronchoalveolar lavage fluid (BALF)

After blood sample collection, mice were euthanized and tracheotomies were performed. The lungs were washed 3 times with instillations of 0.5 mL of PBS via the tracheal cannula. The inflammatory cells analyses was performed as previously described (Olivo et al., 2012).

2.9. Cytokines measurements in BALF by enzyme-linked immunosorbent assay (ELISA)

The levels of the Th2 (IL-4, IL-5 and TSLP), Th1 (tumor necrosis factor-alpha and IL-2) and Th17 (IL17) related cytokines were measured in BALF fluid using ELISA kits (Biolegend, CA, USA; RD Systems, MN, USA) according to manufacturer recommendations

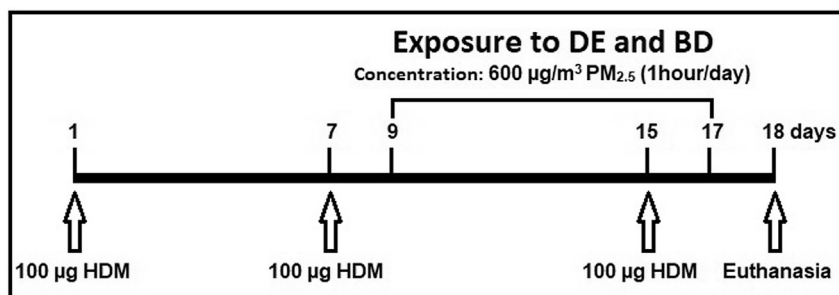


Fig. 2. Experimental design. DE, diesel exhaust; BD, biodiesel exhaust; µg/m³, microgram per cubic meter; PM_{2.5}, Particulate matter smaller than 2.5 µm; HDM, House Dust Mites.

(Rigonato-Oliveira et al., 2018; Vieira et al., 2011).

2.10. Histopathological analysis

The lungs were removed, fixed by intratracheal instillation of 10% buffered formalin solution and paraffin embedded after 24 h. Five- μ m-thick sections were stained with Congo Red for quantifications of eosinophils density in the peribronchial space. The slides were scanned using the Scanner Pannoramic 250 flash III (3DHIS-TECH Ltd; Budapest, Hungary). Analyses were performed using the Case Viewer Software for Windows. Eight peribronchial spaces for each animal were assessed. The results were expressed as cells per area (cells/mm²). All measurements were performed by the same blinded observer.

Silane slides were deparaffinized and hydrated. Next, slides were washed with PBS, and endogenous peroxidase was blocked with oxygenated water (H₂O₂) 10 V 3% and incubated with primary antibody anti-MAC (SC-7885; Santa Cruz) diluted in BSA at a proportion of 1/50.000 overnight. After incubating with secondary antibody Vector Elite – PK-6105, anti-goat (ABC Kit Vectastain, United States), and blocking of the endogenous peroxidase, the slides were revealed with chromogen 3,3 diaminobenzidine (DAB) (Sigma Chemical Co., Saint Louis, Missouri, USA). Next, slides were stained with Harris Hematoxylin (Merck, Darmstadt, Alemanha).

An eyepiece with a coherent system of 50 lines and 100 points with a known area attached to the ocular lens of the microscope was used for conventional morphometry. The density of macrophages was assessed by point counting. Using a 100-point grid with a known area (67.500 μ m² at 1000x magnification) attached to the microscope ocular lens we counted the number of points hitting alveolar tissue in each field. Alveolar tissue area in each field was calculated as the number of points hitting alveolar tissue as a proportion of the total grid area. Data are expressed as macrophage cell density per alveolar area (cells/mm²). Counting was performed in 15 fields of alveolar parenchyma for each animal at a magnification of 1000x.

2.11. Statistical analysis

The distribution data were checked for normality using the Kolmogorov-Smirnov test. Normally distributed variables were reported as the mean $\pm$ SD; non-normally distributed variables as the median and interquartile range. The NO, NO₂ and NO_x concentrations of pollutants measured inside the chamber in real time and values were analyzed by ANOVA test. The curve dose-response was determined using ANOVA repeated measures. The maximum response and AUC of the lung function analyses, cytokines in BALF and lung parenchymal macrophages values were analyzed using ANOVA with post hoc comparisons using Bonferroni's test. Immunoglobulins, IL-17 and peribronchiolar eosinophils values were analyzed using Kruskal-Wallis test, followed by the Mann-Whitney U test. Statistical analyses were performed using SPSS 21.0 (SPSS Inc.[®], Chicago, IL, USA). The level of significance was set to 5%.

3. Results

All non-significant results ($p > 0.05$) are presented in the online supplement (see Table S5, online supplement).

3.1. Exposure assessment and PAH analysis

The concentrations of NO, NO₂ and NO_x were higher in the BD exhaust ($p = 0.000$, $p = 0.016$, $p = 0.000$) and DE exhaust groups ($p = 0.002$, $p = 0.001$, $p = 0.000$) compared to the FA group. Temperature and humidity were similar among groups, and the

patterns are in accordance with the standards of care for rodents (Guide for the Care and Use of Laboratory Animals, 2010) (Table 1).

Table 2 depicts the values of elemental characterization analyzed on filters from the exposure chamber. Diesel fuel had an increased concentration of the elements Ca ($p = 0.002$), Zn ($p = 0.000$), Fe ($p = 0.017$), Na ($p = 0.038$), K ($p = 0.001$), Pb ($p = 0.012$) and S ($p = 0.000$) when compared with Biodiesel fuel.

PAH analysis. Parallel to the samples a spiked sample was prepared to assess the recovery of each analyte and evaluate possible matrix effects. Also, a blank of the extraction procedure was performed as another quality control step. Results for each compound are shown in Table 3. Because Benzo(k)fluorantene, Benzo(j)Fluorantene and Benzo(b)Fluorantene elute with very little difference from each other, they are summed in Tables 3 and 4. Due to high interferences in the diesel sample, some compounds did not present a good chromatographic resolution and are summed in Table 4. The spiked diesel sample was void during preparation and thus is not reported here, but the recovery of the surrogate compounds (2-fluorobiphenyl and terphenyl-D14) are presented as an alternative for quality control purposes.

3.2. Effects of allergic sensitization: HDM vs FA groups

The HDM group presented an increase in the following parameters: (1) serum IgG1 ($p = 0.029$) (Fig. 3, A); (2) AUC ($p = 0.046$) and

Table 1

Average exposure concentrations of pollutants (PM_{2.5}, NO, NO₂ and NO_x) and temperature and relative humidity measured inside the chamber in real time for all experimental groups (FA, BD and DE). The values are expressed as the mean $\pm$ standard deviation (SD).

Parameters	FA	BD	DE
PM _{2.5} (μ g/m ³)	1 $\pm$ 1	622 $\pm$ 121	604 $\pm$ 80
NO (μ g/m ³)	0.6 $\pm$.3	21.5 $\pm$ 17.1 ^a	19.3 $\pm$ 16.1 ^b
NO ₂ (μ g/m ³)	1.2 $\pm$.4	17.3 $\pm$ 18.7 ^a	24.9 $\pm$ 21.3 ^b
NO _x (μ g/m ³)	1.7 $\pm$.6	38.8 $\pm$ 25.8 ^a	44.2 $\pm$ 35.6 ^b
Temperature (°C)	24 $\pm$ 1	23 $\pm$ 1	20 $\pm$ 1
Humidity (%)	81 $\pm$ 7	81 $\pm$ 6	82 $\pm$ 3

FA, filtered air; BD, biodiesel exhaust; DE, diesel exhaust; **Min**, minimum; **Max**, maximum; **PM_{2.5}**, particulate matter with aerodynamic diameter smaller than 2.5 μ m; **NO**, nitric oxide; **NO₂**, nitrogen dioxide; **NO_x**, nitrogen oxides.

^a FA $\neq$ BD [NO, NO_x ($p = 0.000$); NO₂ ($p = 0.016$)].

^b FA $\neq$ DE [NO_x ($p = 0.000$), NO ($p = 0.002$), NO₂ ($p = 0.001$)].

Table 2

Elementary composition of Diesel and Biodiesel exhaust particulate matter as assessed by an Energy Dispersive X-ray Fluorescence Spectrometer. Values are expressed as the median (interquartile range).

Elements (ng/cm ²)	Diesel	Biodiesel	P
Sodium (Na)	172.4 (87)	160.7 (199.8)	0.038
Aluminum (Al)	96.1 (79.90)	105.4 (141)	0.773
Sulfur (S)	66.8 (44.5)	32.4 (36.7)	0.000
Silicium (Si)	54.4 (50.3)	66.1 (192.9)	0.346
Potassium (K)	38.4 (53)	26.1 (58.9)	0.001
Iron (Fe)	29.3 (731.2)	20.6 (629)	0.017
Calcium (Ca)	22.2 (25.4)	15.5 (41.2)	0.002
Copper (Cu)	14.9 (4)	14.4 (14.4)	0.054
Zinc (Zn)	12.2 (11)	8.1 (9.6)	0.000
Nickel (Ni)	9.1 (24.6)	8.8 (22.9)	0.629
lead (Pb)	6.8 (11.4)	5.4 (12.2)	0.012
Titanium (Ti)	5.9 (5.1)	5.5 (6.4)	0.205
Magnesium (Mg)	5.4 (18.5)	4.4 (19.4)	0.701
Chromium (Cr)	4.7 (170.1)	4.7 (149.5)	0.680
Manganese (Mn)	3.4 (40.4)	3.4 (21.4)	0.761
Barium (Ba)	1.8 (1.7)	1.7 (2.4)	0.690
Arsenic (As)	1 (2.3)	1 (2)	0.179
Rubidium (Rb)	1 (0.7)	1 (0.6)	0.739
Vanadium (V)	0.5 (1)	0 (1)	0.801

Table 3Descriptive statistics of polycyclic aromatic hydrocarbon (PAH) for biodiesel fuel. Results are expressed as the mean $\pm$ standard deviation (SD).

Compound	Biodiesel (mg/kg)		
	sample	spike	spike % recovery
Naphtalene	0.0192 $\pm$ 0.0001	0.0198 $\pm$ 0.0015	20.7
2-Fluorobiphenyl (recovery standard)	0.0524 $\pm$ 0.0003	0.0404 $\pm$ 0.0005	42.2
Acenaphthylene	0.0049 $\pm$ 0.0003	0.0398 $\pm$ 0.0003	41.9
Acenaphthene	0.0026 $\pm$ 0.0002	0.0442 $\pm$ 0.0004	46.2
Fluorene	0.0060 $\pm$ 0.0012	0.0523 $\pm$ 0.0010	55.7
Phenanthrene	0.0430 $\pm$ 0.0022	0.0508 $\pm$ 0.0016	53.3
Anthracene	0.0070 $\pm$ 0.0005	0.0571 $\pm$ 0.0055	59.5
Fluorantene	0.0193 $\pm$ 0.0007	0.0636 $\pm$ 0.0049	66.5
Pyrene	0.0199 $\pm$ 0.0007	0.0709 $\pm$ 0.0015	74.8
p-Terphenyl-d14 (recovery standard)	0.0796 $\pm$ 0.0019	0.0744 $\pm$ 0.0010	77.8
Benzo(c)Phenanthrene	0.0019 $\pm$ 0.0002	0.0704 $\pm$ 0.0008	74.0
Chrysene	0.0090 $\pm$ 0.0009	0.0682 $\pm$ 0.0041	71.9
Benzo(a)anthracene	0.0049 $\pm$ 0.0006	0.0740 $\pm$ 0.0035	77.2
Benzo(k)fluorantene + Benzo(j)Fluorantene + Benzo(b)Fluorantene	0.0068 $\pm$ 0.0004	0.1821 $\pm$ 0.0017	64.0
Benzo(e)Pyrene	0.0038 $\pm$ 0.0012	0.0746 $\pm$ 0.0025	78.1
Benzo(a)Pyrene	0.0018 $\pm$ 0.0008	0.0600 $\pm$ 0.0041	62.7
Indene(1,2,3-c,d)Pyrene	0.0020 $\pm$ 0.0001	0.0218 $\pm$ 0.0008	22.9
Dibenz(a,h)Anthracene	0.0020 $\pm$ 0.0001	0.0115 $\pm$ 0.0003	12.1
Benzo(g,h,i)Perylene	0.0020 $\pm$ 0.0001	0.0354 $\pm$ 0.0004	37.6

Table 4Descriptive statistics of polycyclic aromatic hydrocarbon (PAH) for diesel fuel. Results are expressed as the mean $\pm$ standard deviation (SD).

Compound	Diesel (mg/kg)		
	sample	spike	spike % recovery
Naphtalene	8.5 $\pm$ 0.2	—	130
2-Fluorobiphenyl (recovery standard)	0.66 $\pm$ 0.01	—	
Acenaphthylene	3.8 $\pm$ 0.1	—	
Acenaphthene	10.7 $\pm$ 0.9	—	
Fluorene	7.5 $\pm$ 0.7	—	43
Phenanthrene + Anthracene	9.6 $\pm$ 0.1	—	
Fluorantene	6.3 $\pm$ 0.6	—	
Pyrene	22.4 $\pm$ 0.5	—	
p-Terphenyl-d14 (recovery standard)	0.21 $\pm$ 0.01	—	
Benzo(c)Phenanthrene	2.8 $\pm$ 0.1	—	
Chrysene + Benzo(a)anthracene	0.5 $\pm$ 0.02	—	
Benzo(k)fluorantene + Benzo(j)Fluorantene + Benzo(b)Fluorantene	0.3 $\pm$ 0.01	—	
Benzo(e)Pyrene + Benzo(a)Pyrene	0.03 $\pm$ 0.01	—	
Indene(1,2,3-c,d)Pyrene	<0.1	—	
Dibenz(a,h)Anthracene	<0.1	—	0.1 $\pm$ 0.01
Benzo(g,h,i)Perylene	0.1 $\pm$ 0.01	—	

Note: Limit of quantification = 0.1 mg/kg.

maximum Mch response ($p = 0.026$) of Ers (Table 6); (3) tissue peribronchiolar eosinophils density ($p = 0.028$) (Fig. 4, A, E); (4) numbers total cells ($p = 0.020$) eosinophils ($p = 0.038$) (Fig. 6, A and D); (5) IL-5 ($p = 0.002$) and TSLP levels in BALF ($p = 0.046$) (Fig. 7B and C).

3.3. Effects of exposure to BD and DE exhaust: BD and DE vs FA groups

BD exposure alone increased BALF TSLP levels ($p = 0.004$) (Fig. 7, C). No other significant differences were observed for all measured parameters.

DE exposure decreased levels of IgG1 levels ($p = 0.008$) (Fig. 3, A) and increased BALF IL-4 levels ($p = 0.045$) (Fig. 7, A). Ers was also significantly increased in the dose-response curve at baseline ($p = 0.004$), saline ($p = 0.002$), MCh 3 mg/ml ($p = 0.001$), MCh 30 mg/ml, and MCh 300 mg/ml ($p = 0.000$) (Table 5); AUC and maximum Mch response ($p = 0.000$) (Table 6).

3.4. Effects of BD and/or DE exhaust exposure on allergic sensitization: BD+HDM and DE+HDM vs HDM groups

No significant differences were observed in BD+HDM group. DE+HDM group presented reduced levels of serum IgG1 ($p = 0.046$) and IgG2a ($p = 0.043$) (Fig. 3). The density of parenchymal macrophages ($p = 0.037$) (Fig. 5, A, G) and, and BALF total cells ($p = 0.019$), macrophages ($p = 0.034$), lymphocytes ($p = 0.000$), neutrophils ($p = 0.040$) (Fig. 6A and B, C and E), and IL-4 levels ($p = 0.028$) (Fig. 7, A) were increased in DE+HDM group.

3.5. Effects of HDM-sensitization on BD or DE exposure: BD+HDM vs BD and DE+HDM vs DE

There was an increase in the levels of serum IgG1 ($p = 0.000$) (Fig. 3, A), Ers in the maximum Mch response ($p = 0.034$) (Table 6), and peribronchiolar eosinophils density ($p = 0.027$) (Fig. 4, A, F) in the BD+HDM group compared with the BD group.

When compared to DE group, the DE+HDM group presented an increase in the peribronchiolar eosinophils density ($p = 0.010$) (Fig. 4, A, G), parenchymal macrophages ($p = 0.014$) (Fig. 5, A, G),

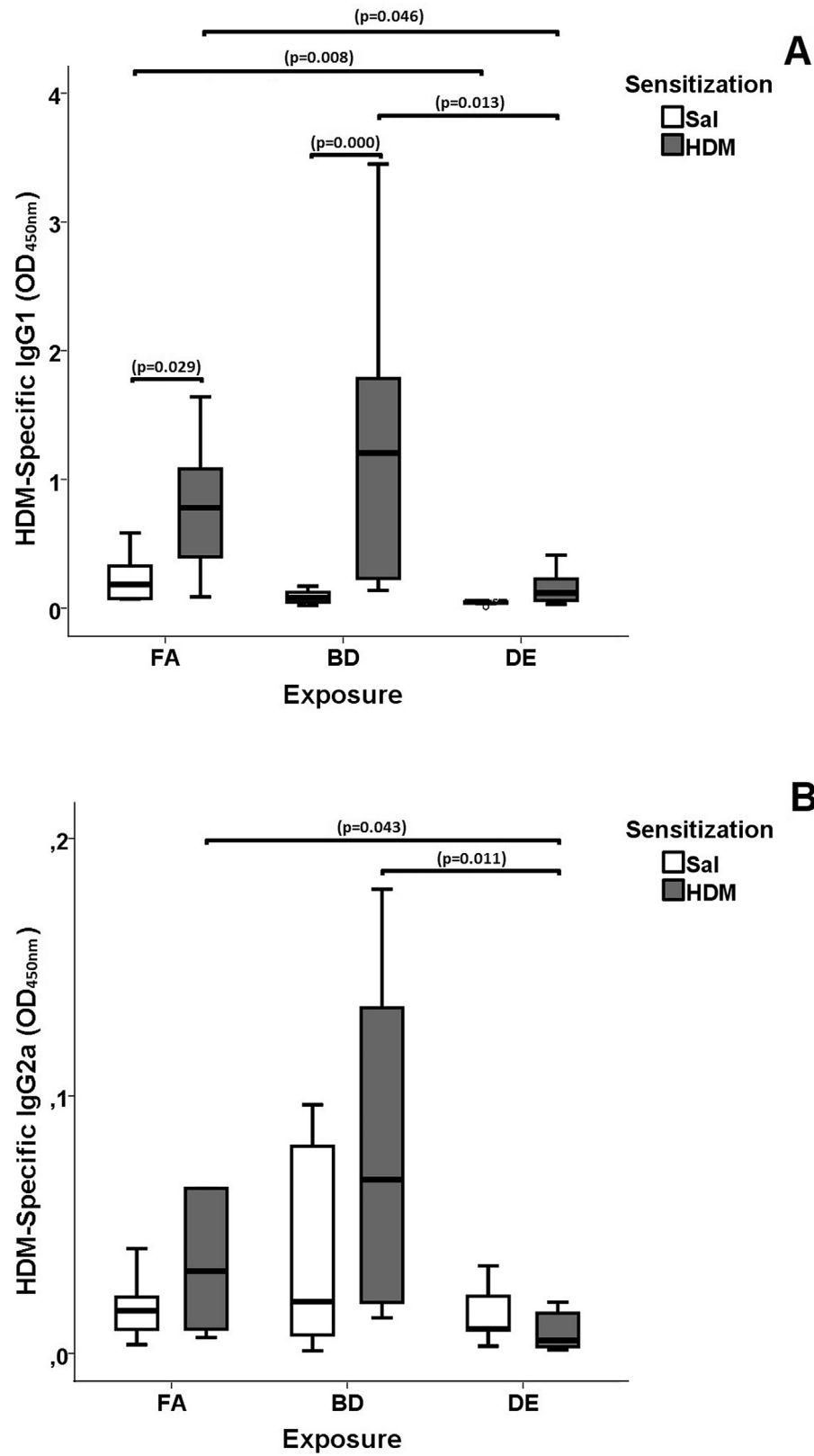


Fig. 3. IgG1 and IgG2a serum levels. **FA**, filtered air; **BD**, biodiesel exhaust; **DE**, diesel exhaust; **Sal**, saline; **HDM**, House Dust Mites. **A.** IgG1, Immunoglobulin 1; **B.** IgG2a, Immunoglobulin 2a.

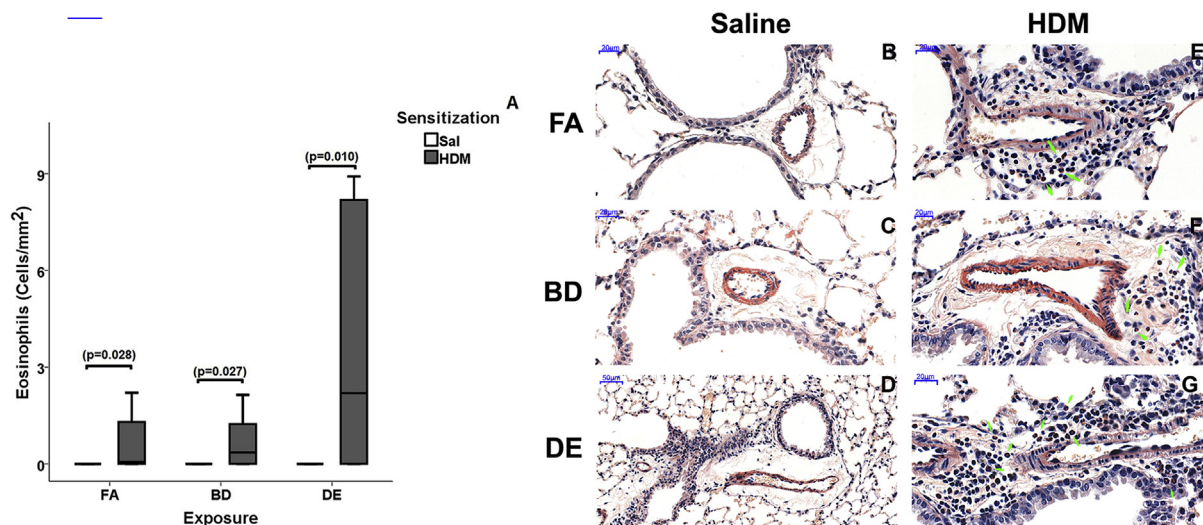


Fig. 4. Representative photomicrographs of peribronchiolar eosinophils. **FA**, filtered air; **BD**, biodiesel exhaust; **DE**, diesel exhaust; **Sal**, saline; **HDM**, House Dust Mites. **A.** Peribronchiolar eosinophils, differences between groups; **B.** FA; **C.** BD; **D.** DE; **E.** HDM; **F.** BD + HDM; **G.** DE + HDM. Scale bars (B, C, E, F, G) 20 µm, (D) 50 µm.

Table 5
Descriptive statistics of dose-response curve to methacholine (MCh) for all experimental groups (FA, HDM, BD, BD+HDM, DE, DE+HDM). Results are expressed as the mean $\pm$ standard deviation (SD).

Parameters	Dose (mg/mL)	FA (n = 11)	HDM (n = 10)	BD (n = 12)	BD + HDM (n = 11)	DE (n = 10)	DE + HDM (n = 11)
Ers (cmH ₂ O.ml ⁻¹)	Baseline	27.48 $\pm$ 1.84	28.91 $\pm$ 2.97	28.50 $\pm$ 2.75	30.40 $\pm$ 3.00	31.70 $\pm$ 3.79*	29.42 $\pm$ 2.49
	Saline	30.19 $\pm$ 2.18	31.95 $\pm$ 3.15	31.47 $\pm$ 3.28	33.96 $\pm$ 3.12	35.22 $\pm$ 3.51*	32.67 $\pm$ 3.54
	3 Mch	32.52 $\pm$ 2.44	34.62 $\pm$ 2.35	34.00 $\pm$ 3.71	36.19 $\pm$ 3.05	38.03 $\pm$ 3.47*	36.80 $\pm$ 4.36
	30 Mch	33.88 $\pm$ 2.63	36.39 $\pm$ 3.12	36.10 $\pm$ 3.94	38.13 $\pm$ 3.23	41.44 $\pm$ 5.18*	38.80 $\pm$ 5.13
	300 Mch	35.29 $\pm$ 3.25	40.14 $\pm$ 4.04	38.85 $\pm$ 4.35	42.88 $\pm$ 4.71	43.20 $\pm$ 3.97*	41.04 $\pm$ 5.85
Rrs (cmH ₂ O.ml ⁻¹ .s)	Baseline	0.70 $\pm$ 0.05	0.69 $\pm$ 0.03	0.71 $\pm$ 0.07	0.72 $\pm$ 0.10	0.71 $\pm$ 0.07	0.71 $\pm$ 0.02
	Saline	0.71 $\pm$ 0.05	0.68 $\pm$ 0.03	0.72 $\pm$ 0.05	0.73 $\pm$ 0.11	0.72 $\pm$ 0.07	0.71 $\pm$ 0.02
	3 Mch	0.71 $\pm$ 0.05	0.70 $\pm$ 0.05	0.73 $\pm$ 0.05	0.74 $\pm$ 0.11	0.73 $\pm$ 0.07	0.73 $\pm$ 0.02
	30 Mch	0.72 $\pm$ 0.06	0.72 $\pm$ 0.07	0.73 $\pm$ 0.04	0.74 $\pm$ 0.08	0.73 $\pm$ 0.04	0.74 $\pm$ 0.03
	300 Mch	0.75 $\pm$ 0.09	0.84 $\pm$ 0.19	0.79 $\pm$ 0.08	0.89 $\pm$ 0.12	0.81 $\pm$ 0.12	0.76 $\pm$ 0.06 [#]

Ers, elastance; **Ers**, Resistance; **FA**, filtered air; **HDM**, House Dust Mites; **BD**, biodiesel exhaust; **BD+HDM**, biodiesel exhaust + HDM sensitized; **DE**, diesel exhaust; **DE+HDM**, diesel exhaust + HDM sensitized. *DE $\neq$ FA [Baseline ($p = 0.004$), Saline ($p = 0.002$), 3 Mch ($p = 0.001$), 30 Mch, and 300 Mch ($p = 0.000$)]; [#]DE+HDM $\neq$ BD+HDM [300 Mch ($p = 0.039$)].

Table 6
Descriptive statistics of area under the curve (AUC) and maximum response for all experimental groups (FA, HDM, BD, BD+HDM, DE, DE+HDM). Results are expressed as the mean $\pm$ standard deviation (SD).

Parameters		FA (n = 11)	HDM (n = 10)	BD (n = 12)	BD + HDM (n = 11)	DE (n = 10)	DE + HDM (n = 11)
AUC	Ers	10244 $\pm$ 765	11399 $\pm$ 896*	11070 $\pm$ 1212	11950 $\pm$ 1119	12510 $\pm$ 1298**	11810 $\pm$ 1609
	Rrs	218 $\pm$ 19	230 $\pm$ 37	229 $\pm$ 17	240 $\pm$ 25	229 $\pm$ 22	223 $\pm$ 13
Maximum response	Ers (cmH ₂ O.ml ⁻¹)	36 $\pm$ 2	40 $\pm$ 4*	39 $\pm$ 4	43 $\pm$ 5 [#]	44 $\pm$ 5**	41 $\pm$ 6
	Rrs (cmH ₂ O.ml ⁻¹ .s)	0.77 $\pm$ 0.09	0.84 $\pm$ 0.19	0.79 $\pm$ 0.08	0.90 $\pm$ 0.12	0.82 $\pm$ 0.12	0.77 $\pm$ 0.06

Ers, elastance; **Ers**, Resistance; **FA**, filtered air; **HDM**, House Dust Mites; **BD**, biodiesel exhaust; **BD+HDM**, biodiesel exhaust + HDM sensitized; **DE**, diesel exhaust; **DE+HDM**, diesel exhaust + HDM sensitized. *HDM $\neq$ FA [AUC ($p = 0.046$); Maximum response ($p = 0.026$)]; **DE $\neq$ FA ($p = 0.000$); [#]BD+HDM $\neq$ BD ($p = 0.034$).

and in the number of total cells, macrophages ($p = 0.000$), eosinophils ($p = 0.007$), lymphocytes, and neutrophils ($p = 0.001$) (Fig. 6) in the BALF.

3.6. DE+HDM vs BD+HDM

In comparison with the BD+HDM group, the DE+HDM group had lower levels of serum IgG1 ($p = 0.013$) and IgG2a ($p = 0.011$) (Fig. 3). Rrs was also significantly decreased in the dose-response curve in the MCh 300 mg/ml dose ($p = 0.039$) (Table 5).

In the BALF, DE+HDM group presented higher levels of parenchymal macrophages ($p = 0.019$) (Fig. 5, A, F, G), BALF total cells ($p = 0.007$), eosinophils ($p = 0.010$), and macrophages ($p = 0.002$)

when compared to the BD+HDM group (Fig. 6A and B and D).

4. Discussion

In this study, we used a model of acute allergic sensitization to describe the effects of biodiesel and diesel exhaust exposure upon allergen challenge. HDM-sensitized animals presented elevated IgG1 serum levels, increased lung elastance, BALF and tissue eosinophilia with increased BALF levels of IL-5 and TSLP. Eight-day DE exposure alone increased lung elastance and BALF IL-4 levels, whereas BD exposure alone increased BALF TSLP levels. Whereas BD exposure did not influence any parameter after HDM challenge, DE-exposed animals presented increased neutrophilic (BALF) and

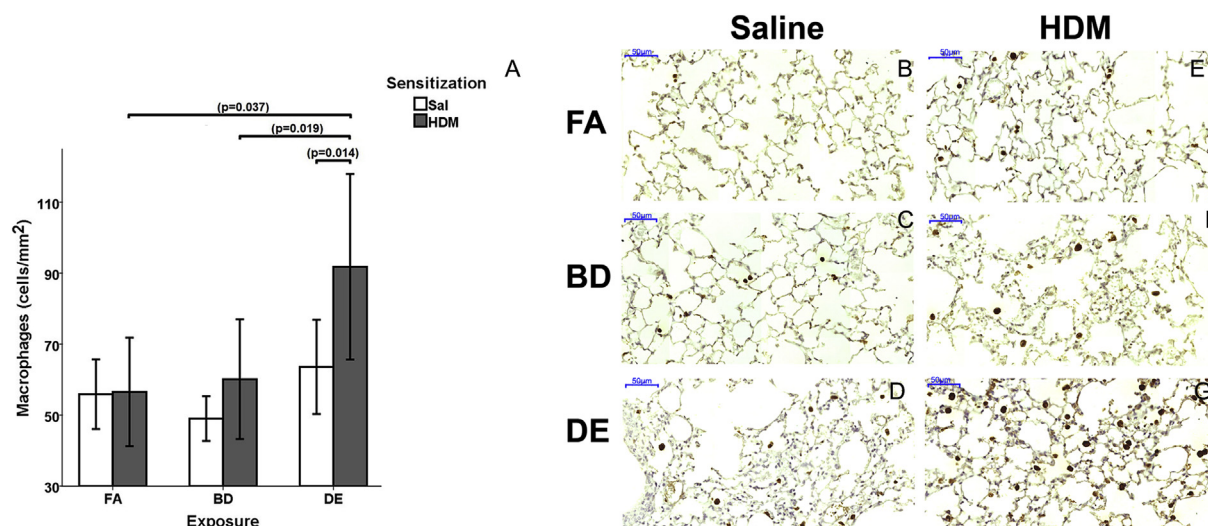


Fig. 5. Representative photomicrographs of alveolar macrophages, immunostained in brown. **FA**, filtered air; **BD**, biodiesel exhaust; **DE**, diesel exhaust; **Sal**, saline; **HDM**, House Dust Mites. **A**. Parenchymal macrophages, differences between groups; **B**. FA; **C**. BD; **D**. DE; **E**. HDM; **F**. BD +HDM; **G**. DE+HDM. Scale bars (B, C, D, E, F, G) 50 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

macrophagic inflammation (BALF and lung tissue), as well as further increased IL-4 and decreased IgG1 and IgG2 levels when compared to the HDM group.

Our results indicate that soy/tallow BD exposure in previously sensitized animals did not influence allergic parameters. Our results are in accordance with [Gavett et al. \(2015\)](#), who that showed that, when compared to diesel exposure, soy BD exposure during allergic sensitization did not elicit increased responses.

We have previously shown that an acute, single exposure of 600–1200 µg/m³ MP_{2.5} BD has similar deleterious effects on pulmonary and cardiovascular parameters in healthy mice as diesel ([Brito et al., 2010](#); [de Brito et al., 2018](#)). Additionally, pharyngeal instillation ([Yanamala et al., 2013](#)) and inhalation exposure ([Shvedova et al., 2013](#)) showed a higher lung inflammatory response after BD exposure compared to DE exposure. Models of chronic exposure to low concentrations of biodiesel and diesel have shown modest alterations in pulmonary parameters, with diesel causing more pulmonary injury and B100 more acute vascular effects ([Bass et al., 2015](#)). It is interesting to note that in the model of allergic inflammation the deleterious effects of BD were not apparent ([Gavett et al., 2015](#)).

It is possible that the different PM compositions and PAH concentrations in both fuels could account for these results. In our study, PM_{2.5} from BD contained smaller concentrations of several elements, such as S, Na, K, Ca, Fe, Zn and Pb. Previous studies in asthmatic children have related the association of asthma prevalence and Zn in PM₁₀ at the birth address. Asthma symptoms and allergic sensitization were associated with Copper and Iron in PM_{2.5} samples at the current address of children ([Gehring et al., 2015](#)). In Santiago (Chile), asthma symptoms in children were associated with 7-day exposures for the PM_{2.5} components OC, K, S, Se, V ([Prieto-Parra et al., 2017](#)). Our data showed much higher concentrations of different PAH in diesel than in biodiesel, which could also explain the differences in allergic responses of the different animal groups. Accordingly, previous data have linked several adverse effects of PAH exposure in pediatric asthma, which may be mediated by allergenic or oxidative stress pathways ([Karimi et al., 2015](#); [Wang et al., 2017](#)).

The HDM model in this study was previously used by [Vieira et al. \(2011\)](#). In our study, HDM exposed animals presented increased expression of IgG1, BALF and tissue peribronchiolar eosinophilia

and increased IL-5 levels in BALF, indicating the adequacy of the model. BD and DE exposure alone over 8 days did not significantly alter inflammatory parameters, but rather induced the expression of different Th2 related cytokines in BALF (IL-4 in DE and TSLP in BD). These data support the idea that biodiesel and diesel exposure can modulate the immunological system towards Th2 type inflammation ([Meldrum et al., 2017](#)). Fuel exposition did not alter significantly BALF cytokine measurements in sensitized animals, except for IL-5, which decreased in sensitized DE animals in relation to their sensitized only controls. [Gavett et al. \(2015\)](#) could also not find any BALF or lung tissue differential inflammation between BD- and DE-exposed animals but detected increases in IL-17 and other Th2 cytokine secretions in peribronchiolar LNs in DE-exposed mice.

TSLP secretion by lung epithelial cells has been implicated as a possible pathway linking diesel exposure and (Th2) allergic inflammation ([Segawa and Hirasawa, 2014](#)). In our study, biodiesel (but not diesel) exposure increased TSLP expression in relation to saline controls, that did not further increase with previous HDM exposure. The reasons for this finding were not clear. It is possible that HDM exposure alone caused an increase in TSLP reaching plateau levels, not further significantly increased by BD exposure.

There was a significant increase in macrophages, neutrophils and lymphocytes in the BALF of sensitized mice exposed to DE compared to their related HDM controls. There was no further increase in BALF or tissue eosinophils and a decrease in serum levels of IgG1/2. The contribution of macrophages to the pathogenesis of asthma is still unclear. An imbalance between type 1 and 2 macrophages might be involved in the progression of asthma ([Moreira and Hogaboam, 2011](#)), while the literature shows that complete depletion of alveolar macrophages significantly increases inflammation ([Bang et al., 2011](#)). [Zastona et al. \(2014\)](#) showed that resident alveolar macrophages in a murine model of asthma suppress allergic lung inflammation, while recruited macrophages promote allergic lung inflammation. Diesel exhaust promoted an increase in IL-8 in M1 macrophages and IL-10 in M2 macrophages ([Jaguin et al., 2015](#)). We have not studied macrophage polarization or proliferation, but it is plausible to suppose that the decrease in IgG1/2 and IL-5 levels and no further increase in eosinophils in DE-exposed animals could be related to the damping properties of certain macrophage types in relation to allergic inflammation. The role of

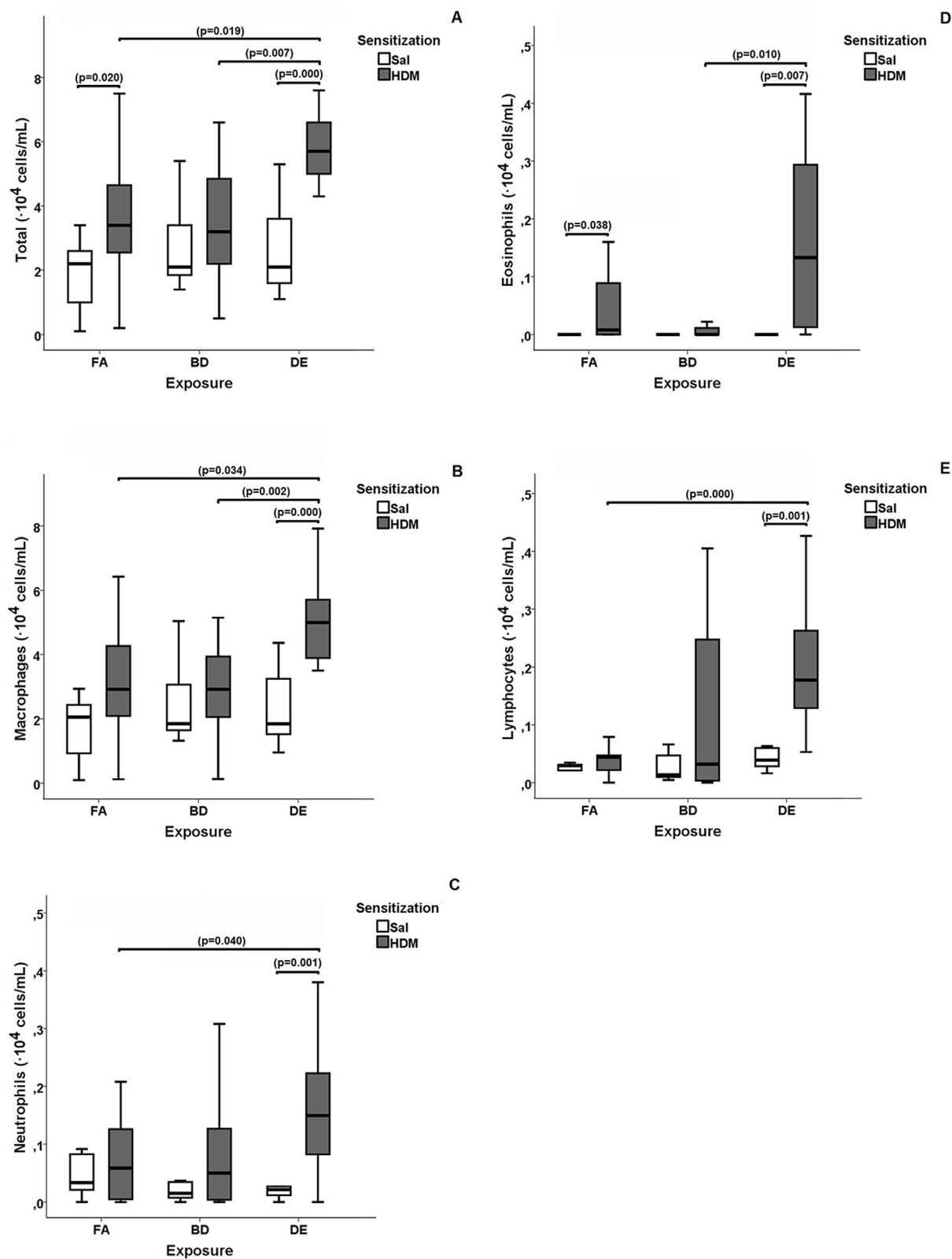


Fig. 6. Inflammatory cells in bronchoalveolar lavage fluid (BALF). FA, filtered air; BD, biodiesel exhaust; DE, diesel exhaust; Sal, saline; HDM, House Dust Mites. A. Total cells; B. Macrophages; C. Neutrophils; D. Eosinophils; E. Lymphocytes.

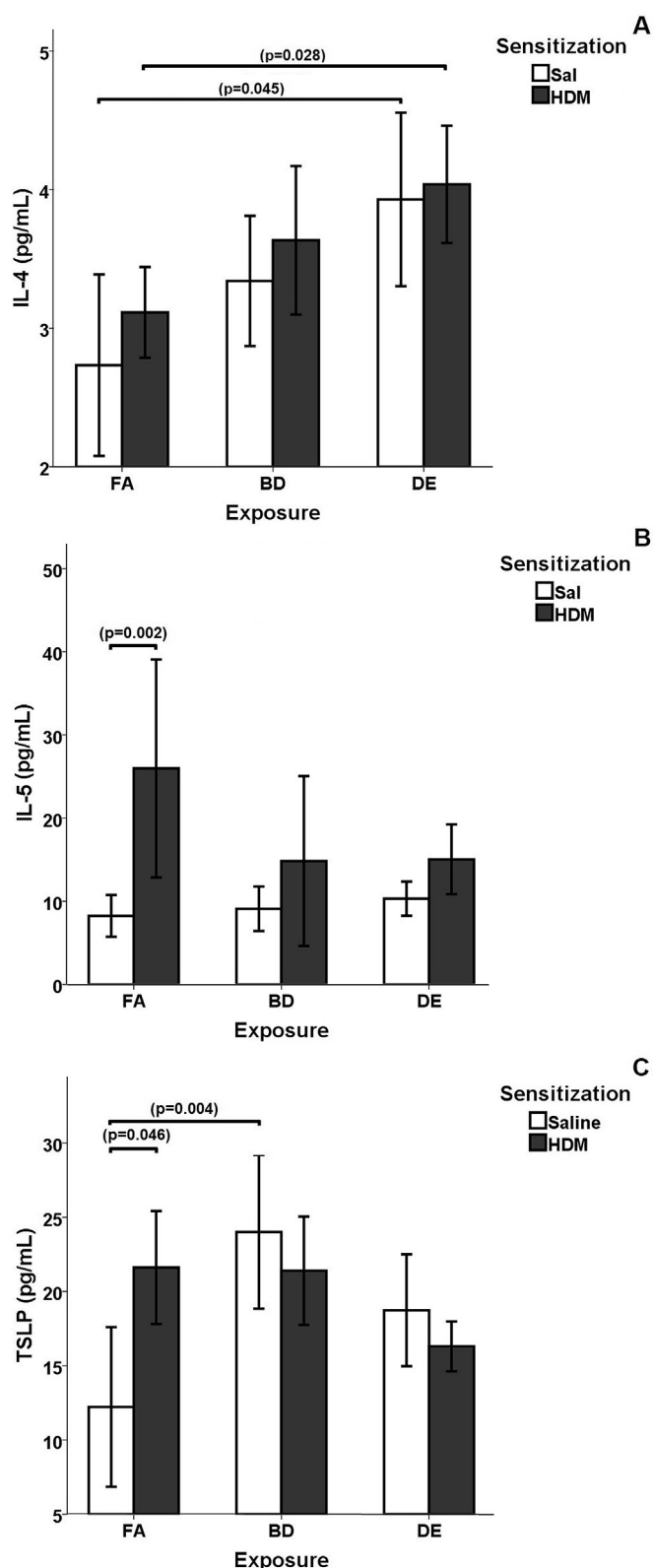


Fig. 7. Cytokine levels in BALF. FA, filtered air; BD, biodiesel exhaust; DE, diesel exhaust; Sal, saline; HDM, House Dust Mites. A. IL-4, Interleukin-4; B. IL-5, Interleukin-5; C. TSLP, Thymic Stromal Lymphopoietin.

environmental factors in asthma severity is not clear, and certain severe non-allergic asthma phenotypes have been linked to neutrophilic/eosinophilic inflammation (Ray and Kolls, 2017). Our data indeed suggest that vehicular pollution might contribute to the neutrophilic asthma phenotype. In studies with bronchial biopsies of asthmatics, acute DE induced the presence of more CD4, IL-4 and neutrophils in the airway mucosa, but not eosinophils or mast cells (Hosseini et al., 2015).

There are some limitations in this study. We could not find significant differences in NO, NO₂ and NO_x emissions between fuels. A review on the effects of BD on NO_x emissions has shown that in some experimental settings the emissions of NO_x were not higher when compared to diesel. NO emissions derived from BD vary according to biodiesel composition, type of engine, injection timing, ignition delay and other combustion phenomena (Hoekman and Robbins, 2012). We had not analyzed PAH and elemental carbon in the diesel/biodiesel exhausts which limits our evidence about the differential biological responses in diesel vs biodiesel exposures. However, in a previous study of our group, using a similar model of fuel exhaustion, we have shown that concentrations of PAH and BC are substantially decreased when diesel and B100 (biodiesel 100%) were compared (97.8% PAH and BC 38.65%) (Brito et al., 2010). Other studies confirmed similar results (Karavalakis et al., 2017; Mutlu et al., 2015).

We used BD100 (100% biodiesel, no mixture with diesel) for the experiments, which may not mimic real life exposures. Adverse health effects of the two fuel types may also rely on the variation in production routes, fuel sources, blends, engines, engine speed and load. We have not fully investigated the mechanisms by which DE affects allergic inflammation, especially epigenetic changes and expression of microRNAs (Clifford et al., 2017; Rider et al., 2018; Zhang et al., 2018).

Taken together, our data indicate more pulmonary adverse effects of DE over BD exhaust exposure in allergic sensitized animals, but clinical studies in asthmatics are still lacking. The development of cleaner and more sustainable fuels should not proceed apart from the knowledge on their effects on human health, particularly in more vulnerable populations such as asthmatics.

Conflicts of interest

No conflicts of interest, financial or otherwise, are declared by the author(s).

Main finding of the work

Diesel exhaust cause more pulmonary adverse effects than the biodiesel exhaust exposure in allergic sensitized animals.

Acknowledgements

This work was supported by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, São Paulo, Brazil, 2008/57717-6) and the National Institute of Integrated Analysis of Environmental Risk (INAIRA, São Paulo, Brazil, 573813/2008-6). Thais Mauad is funded by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2019.06.085>.

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**Inflammatory and functional responses after (bio)diesel
exhaust exposure in allergic sensitized mice. A comparison
between diesel and biodiesel**

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Supplementary material

2. Material and methods

2.2. Fuels and exposure system

Table S1. Physical properties of the biodiesel fuel used in this study.

Determination	Method	ANP Specification	Result
Aspect	NBR 16048	LII ⁽¹⁾	LII ⁽²⁾
Specific mass to 20°C (kg/m ³)	NBR 14065	850 - 900	877.7
Kinematic viscosity to 40°C (mm ² /s)	NBR 10441	3 - 6	4.374
Tenor of water (mg/kg)	ASTM 6304	maximum 200	170
Total contamination (mg/kg)	EN 12662	maximum 24	15.9
Flash point (°C)	NBR 14598	minimum 100	136
Tenor of éster (%m/m)	EN 14103	minimum 96.5	98.6
Total sulfur (mg/kg)	ASTM D5453	maximum 10	5.5
Tenor of Sodium + Potassium (mg/kg)	NBR 15556	maximum 5	<0.8
Tenor of Calcium +	NBR 15553	maximum 5	<2.0
Tenor of phosphorus (mg/kg)	NBR 15553	maximum 10	<1.0
Clogging point (°C)	NBR 14747	maximum 14	4
Acid index (mg KOH/g)	ASTM D664	maximum 0.50	0.37
Free glycerin (%m/m)	ASTM D6584	maximum 0.02	0.009
Total glycerin (%m/m)	ASTM D6584	maximum 0.25	0.170
Monoglycerides (%m/m)	ASTM D6584	maximum 0.70	0.487
Diglycerides (%m/m)	ASTM D6584	maximum 0.20	0.177
Triglycerides (%m/m)	ASTM D6584	maximum 0.20	0.084
Oxidation stability to 110°C (h)	EN 14112	minimum 8	13.6

ANP, National Petroleum Agency; **NBR**, Brazilian Standards; **ASTM**, American Society for Testing and Materials; **EN**, European Standard.

* Resolution ANP nº 45/2014. Establishes the specification of biodiesel and the obligations concerning quality control to be met by the various economic agents that commercialize the product throughout the national territory. Source: ANP, 2014.

(1) ILL – clear and free of impurities

(2) Assay carried out at 28°C

Table S2. Physical properties of the diesel fuel used in this study.

Determination	Method	ANP Specification*	Result
Aspect	NBR 14954	Clean and water free and particulate matter	Turbid and water free and particulate matter
Color visual	Visual	yellow	yellow
Color ATSM	NBR 14483	-	color ASTM 1.5
Tenor of biodiesel (%vol./vol.)	NBR 15568	7.5 – 8.5	8.0
Sulfur (mg/kg)	ASTM D5453	maximum 10	5.5
Distillation	NBR 9619	-	-
Distillation 10% vol. Recovered (°C)	NBR 9619	minimum 180	199.6
Distillation 50% vol. Recovered (°C)	NBR 9619	245 - 295	261.2
Distillation 85% vol. Recovered (°C)	NBR 9619	-	319.7
Distillation 90% vol. Recovered (°C)	NBR 9619	-	328.9
Distillation 95% vol. Recovered (°C)	NBR 9619	maximum 370	342.0
Specific mass to 20°C (kg/m ³)	NBR 14065	815 -850	833.1
Flash point (°C)	NBR 14598	minimum 38	59.0
Cinematics viscosity to 40°C (mm ² /s)	NBR 10441	2 - 5	2.466
Clogging point (°C)	NBR 14747	-	19
Carbon residue Ramsbottom in 10% of the end distillation (%m/m)	NBR 14318	maximum 0.25	<0.15
Ashes (%m/m)	NBR 9842	maximum 0.010	0.002
Corrosivity to copper (3 hours 50°C)	NBR 14359	maximum 1	1A
Tenor of water Karl-Fischer (mg/kg)	ASTM D6304	maximum 200	282
Total contamination (mg/kg)	EN 12662	maximum 24	14
Tenor of water and sediment (%vol./vol.)	ASTM D 2709	maximum 0.05	0.02
Lubricity (μ)	ASTM D6079	maximum 520	185
Electric conductivity (pS/m)	ASTM D2624	minimum 25	1480

ANP, National Petroleum Agency; **NBR**, Brazilian Standards; **ASTM**, American Society for Testing and Materials; **EN**, European Standard.

* Resolution ANP nº 50/2013. Establishes specifications for road use diesel and the obligations concerning quality control to be met by the various economic agents that commercialize the product throughout the national territory. Source: ANP, 2013.

Supplement S3. Polycyclic aromatic hydrocarbon (PAH) analysis

Analytes and analytical standards

The following analytes were determined in both diesel and biodiesel samples:

- Naphtalene, CAS: 91-20-3
- Acenaphtylene, CAS: 208-96-8
- Acenaphtene, CAS: 83-32-9
- Fluorene, CAS: 86-73-7
- Phenantrene, CAS: 85-01-8
- Anthracene, CAS: 120-12-7
- Fluorantene, CAS: 206-44-0
- Pyrene, CAS: 129-00-0
- Benzo[a]anthracene, CAS: 56-55-3
- Chrysene, CAS: 218-01-9
- Benzo[b]fluorantene, CAS: 205-99-2
- Benzo[k]fluorantene, CAS: 207-08-9
- Benzo[j]fluorantene, CAS: 205-82-3
- Benzo[e]pyrene, CAS: 192-97-2
- Benzo[a]pyrene (BaP), CAS: 50-32-8
- Indene[1,2,3-cd]pyrene, CAS: 193-39-5
- Dibenz[a,h]anthracene, CAS: 53-70-3
- Benzo[g,h,i]perylene, CAS: 191-24-2

Naphtalene-d8 (CAS: 1146-65-2), Acenaphtene-d10 (CAS: 15067-26-2), Phenantrene-d10 (CAS: 1517-22-2, Chrysene-d12 (CAS: 1719-03-5) and

Perylene-d12 (CAS: 1520-96-3) were used as internal standards and 2-Fluorobiphenyl and D14-p-terphenyl were used as recovery standards.

All analytical standards were prepared by the dilution and/or combination of commercial standards purchased from Accustandard Inc. (CT, USA) and were prepared by dilution in isooctane (Sigma-Aldrich Co., MO, USA).

Sample extraction and purification methodology

Biodiesel and diesel extraction: 5 g of the biodiesel sample and 1 g of diesel was weighed and fortified with a known amount of the recovery standards (0,1 µg/g of sample). Then 100 mL of alcoholic potassium hydroxide solution (prepared with 80 g of Potassium hydroxide (Química Moderna Ind., SP, Brazil) dissolved in 40 mL of water and make up to 600 mL with methanol (Bio-Grade Chem., CA, USA)) was added. This mixture was left in a water bath for circa 60 minutes for the complete hydrolysis of the biodiesel and then it was extracted with three portions of 100 mL toluene (Honeywell International Inc., MI, USA). The toluene extract was then washed with three portions of 50 mL methanol: water (2:1) and collected in a round bottomed flask after passing through a paper filter with sodium sulfate.

Cleanup for biodiesel extract: The extract was concentrated to circa 1 mL in a rotary evaporator (Buchi model Rotavapor R-215) and quantitatively transferred to a glass column filled with 3.75 g of Florisil® (Sigma-Aldrich Co., MO, USA) hydrated with 7 % H₂O and pre-conditioned with 10 mL hexane. The column was eluted with 6.5 mL hexane and then with 19 mL 5 % diethyl ether in hexane. All the eluate was collected in a round bottomed flask and re-

concentrated to 1 mL at 37,5 °C under nitrogen. Finally, it was added 1 µg/mL of the internal standards to the extract and the GCMS analysis was performed.

Cleanup for diesel extract: The extract was concentrated to circa 5 mL in a rotary evaporator (Buchi model Rotavapor R-215), quantitatively transferred to a 5 mL volumetric flask and filled to the mark with hexane. Then a glass column filled with 1 g of Florisil® (Sigma-Aldrich Co., MO, USA) was pre-conditioned with 2 mL hexane. An aliquot of 1 ml of the extract was loaded to the column and washed with a portion of 2.8 mL hexane and then the PAHs were eluted with a portion of 3.0 ml dicloromethane:hexane (1:5). All the eluate was collected in a round bottomed flask and re-concentrated to 1 mL at 37,5 °C under nitrogen. Finally, it was added 1 µg/mL of the internal standards to the extract and the GCMS analysis was performed.

CGMS analysis

The following conditions were used for the CGMS analysis:

Instrument: gas chromatograph coupled to mass spectrometer, Agilent Model 7890A(GC)+5975C(MS).

Column: CP-SIL PAH-CB Ultimetel (25 m x 0,25 mm x 0,12 µm)

Injector: 300 °C

Carrier gas: Helium

Injection mode: Pulsed splitless (pulse of 20 psi for 1 minute)

Injection volume: 2 µL

Oven program: initial 60°C for 1 min.

10 °C/min. up to 250 °C

5 °C/min. up to 310 °C, maintained for 6 min.

Transfer line: 350 °C

Ion source temp.: 230 °C

Ionization mode: Electron impact, 70 eV

Analysis mode: Selected Ion Monitoring (SIM)

Monitored Ions:

Compound	Quantification	Qualification ion	Qualification ion
Naphtalene-d8	136	134	108
Acenaphtene-d10	164	162	160
Phenantrene-d10	188	184	189
Chrysene-d12	240	236	241
Perylene-d12	264	260	265
Naphtalene	128	127	102
2-Fluorobyphenyl (recovery standard)	172	171	170
Acenaphthilene	152	151	150
Acenaphtene	153	154	152
Fluorene	166	165	167
Phenantrene	178	176	179
Anthracene	178	176	179
Fluorantene	202	200	203
Pyrene	202	200	203
p-Terphenyl-d14	244	122	245
Benzo(c)Fluorantene	228	227	226
Chrysene	228	226	229
Benzo(a)anthracene	228	226	229
Benzo(k)fluorantene	252	250	253
Benzo(j)Fluorantene	252	250	253
Benzo(b)Fluorantene	252	250	253
Benzo(e)Pyrene	252	250	253
Benzo(a)Pyrene	252	250	253

Indene(1,2,3-c,d)Pyrene	276	277	274
Dibenz(a,h)Anthracene	278	276	279
Benzo(g,h,i)Perylene	276	138	277

2.5. Study design

Table S4. Number of animals used in each procedure. The total number animals were 11 per group, with 12 animals in the BD group.

Parameters	Number of the animals					
	FA	HDM	BD	BD+HDM	DE	DE+HDM
Lung mechanical	11	10	12	11	10	11
Immunoglobulins (IgE, IgG1, IgG2a)	9	10	10	8	9	11
BALF	9	11	12	11	11	11
Cytokines	9	11	11	9	9	9
Lung parenchymal macrophages and peribronchiolar eosinophils	11	11	12	11	11	11

FA, filtered air; **HDM**, House Dust Mites; **BD**, biodiesel exhaust; **BD+HDM**, biodiesel exhaust + HDM sensitized; **DE**, diesel exhaust; **DE+HDM**, diesel exhaust + HDM sensitized; **BALF**, bronchoalveolar lavage.

3. Results

Non-significant results

Table S5. Non-significant results ($p>0.05$), which are expressed as the mean $\pm$ standard deviation (SD) or median (interquartile range) from different parameters analyzed in this study.

Variable	FA	HDM	BD	BD+HDM	DE	DE+HDM
HDM-specific IgE (OD450nm)	0.02 (0.08)	0.01 (0.12)	0.01 (0.03)	0.03 (0.08)	0.01 (0.05)	0.02 (0.06)
IL-2 (pg/mL)	32.55 $\pm$ 23.53	36.23 $\pm$ 17.73	37.55 $\pm$ 25.17	31.00 $\pm$ 10.37	28.58 $\pm$ 17.09	18.34 $\pm$ 8.31
IL-17 (pg/mL)	3.455 (45.13)	4.44 (56.87)	9.70 (9.26)	14.42 (31.75)	18.70 (43.11)	8.89 (80.32)
TNF- α (pg/mL)	24.47 $\pm$ 9.94	30.95 $\pm$ 12.87	22.72 $\pm$ 10.15	32.19 $\pm$ 8.28	30.17 $\pm$ 10.11	28.59 $\pm$ 4.32

Note. **FA**, filtered air; **HDM**, House Dust Mites; **BD**, biodiesel exhaust; **BD+HDM**, biodiesel exhaust + HDM sensitized; **DE**, diesel exhaust; **DE+HDM**, diesel exhaust + HDM sensitized; **IgE**, Immunoglobulin E; **IL-2**, Interleukin-2; **IL-17**, Interleukin-17; **TNF- α** , Tumor necrosis factor-alpha.