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The influence of 5-lipoxygenase on cigarette smoke-induced emphysema in mice

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ABSTRACT

Background: Pulmonary emphysema is characterized by the loss of lung architecture. Our hypothesis is that the inhibition of 5-lipoxygenase (5-LO) production may be an important strategy to reduce inflammation, oxidative stress, and metalloproteinases in lung tissue resulting from cigarette smoke (CS)-induced emphysema.
Methods: 5-LO knockout (129S2-Alox5^{tm1Fun}/J) and wild-type (WT) mice (129S2/SvPas) were exposed to CS for 60 days. Mice exposed to ambient air were used as Controls. Oxidative, inflammatory, and proteolytic markers were analyzed.
Results: The alveolar diameter was decreased in CS 5-LO$^{-/-}$ mice when compared with the WT CS group. The CS exposure resulted in less pronounced pulmonary inflammation in the CS 5-LO$^{-/-}$ group. The CS 5-LO$^{-/-}$ group showed leukotriene B4 values comparable to those of the Control group. The expression of MMP-9 was decreased in the CS 5-LO$^{-/-}$ group when compared with the CS WT group. The expression of superoxide dismutase, catalase, and glutathione peroxidase were decreased in the CS 5-LO$^{-/-}$ group when compared with the Control group. The protein expression of nuclear factor (erythroid-derived 2)-like 2 was reduced in the CS 5-LO$^{-/-}$ group when compared to the CS WT group.
Conclusion: In conclusion, we show for the first time that 5-LO deficiency protects 129S2 mice against emphysema caused by CS. We suggest that the main mechanism of pathogenesis in this model involves the imbalance between proteases and antiproteases, particularly the association between MMP-9 and TIMP-1.
General significance: This study demonstrates the influence of 5-LO mediated oxidative stress, inflammation, and proteolytic markers in CS exposed mice.

1. Introduction

Pulmonary emphysema is considered the major pulmonary chronic obstructive disorder included in the COPD group of diseases and is characterized by the abnormal enlargement of the airspaces with destruction and disorder of the lung architecture [1]. Due to the large number of individuals who smoke and consume other forms of tobacco, the World Health Organization (WHO) considers smoking to be an epidemic that causes enormous health problems [2]. Currently, smoking is the primary factor for the development of emphysema [3] because the inhalation of cigarette smoke (CS) promotes protease/antiprotease imbalance, oxidative stress, alveolar cellular apoptosis, remodeling of the extracellular matrix [4], and the generation of reactive oxygen species (ROS), which cause lesions on the structure of lipids, carbohydrates, proteins, and DNA [5]; each of these damaged factors may contribute to the complexity of the disease [6].

The imbalance in COPD oxidant/antioxidant and protease/antiprotease mechanisms causes tissue damage that contributes to the breakdown of the pulmonary extracellular matrix [7,8]. Metalloproteinases (MMPs) comprise a family of enzymes that have the ability to degrade extracellular matrix components and are important in processes of embryonic development, growth, and tissue repair and remodeling. These enzymes also play an important role in the cell migration of both tumor cells and connective tissue cells and may act in the regulation of molecules such as cytokines and growth factors [9]. In addition, ROS can affect the activity of 5-lipoxygenase (5-LO), an enzyme that plays a crucial role in the inflammatory response [10]. 5-LO is present

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Table 1
The primers used in quantitative real-time PCR.

Gene	Primer	Sequence (5′→3′)	Amplicon size
iNOS	Sense	TGGTGGTGACAAGCACATTT	119 bp
	Antisense	AAGGCCAAACACAGCATACC	
MMP-9	Sense	CGTCGTGATCCCCACTTACT	225 bp
	Antisense	AACACACAGGGTTTGCCTTC	
MMP-12	Sense	TTTCTTCCATATGGCCAAGC	142 bp
	Antisense	ATGCTCCTGGGATAGTGTGG	
TIMP-1	Sense	CATGGAAAGCCTCTGTGGAT	132 bp
	Antisense	CTCAGAGTACGCCAGGGAAC	
TIMP-4	Sense	ACCTCCGGAAGGAGTACGTT	136 bp
	Antisense	TTATCTGGCAGCAACACAGC	
SOD	Sense	TCAATGGTGGGGGACATATT	99 bp
	Antisense	GCTTGATAGCCTCCAGCAAC	
CAT	Sense	CCTCGTTCAGGATGTGGTTT	130 bp
	Antisense	TCTGGTGATATCGTGGGTGA	
GPx	Sense	ATTCTCCACACCCTGTTTCG	112 bp
	Antisense	GATTCCTGGAAGGTGGTCAA	
Nrf2	Sense	AGCCTCTGTCACCAGCTCA	108 bp
	Antisense	ATGGGGCTTTTTGATGACC	
HPRT-1	Sense	GCTACAGCTTCACCACCACA	112 bp
	Antisense	TCTCCAGGGAGGAAGAGGAT	

iNOS, inducible nitric oxide synthase; MMP-9, matrix metalloproteinase 9; MMP-12, matrix metalloproteinase 12; TIMP-1, tissue inhibitor of matrix metalloproteinase 1; TIMP-4, tissue inhibitor of matrix metalloproteinase 4; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; Nrf2, Nuclear factor (erythroid-derived 2)-like 2; HPRT-1, hypoxanthine phosphoribosyl-transferase 1.

in the nucleus and cytosol of non-activated cells [11], and it is responsible for catalyzing the conversion of arachidonic acid to leukotrienes, which are lipid derivatives involved in the immune system's response to inflammatory diseases though leukotriene release [12,13].

Our hypothesis is that the direct intervention on leukocyte migration in cigarette smoke-induced pulmonary emphysema via the inhibition of 5-LO production may be an important strategy to reduce inflammation, oxidative stress, and metalloproteinases in lung tissue. Therefore, our aim was to investigate the role of 5-LO in the inflammatory process of pulmonary emphysema induced by cigarette smoke.

2. Material and methods

2.1. Chemicals and animals

All chemicals were purchased from the Sigma-Aldrich Chemical Company (St Louis, MO, USA) unless specified otherwise. Male (8 weeks old; 20–24 g) 129S2-Alox5^{tm1Fun}/J (5-lipoxygenase knockout) mice and their littermates 129S2/SvPas [14] were purchased from CECAL/IOC (Laboratory Animal Facility — Oswaldo Cruz Institute, Rio de Janeiro, Brazil). The mice were housed 15 per cage in a room with a controlled environment, a 12-h light/12-h dark cycle (lights off at 7 pm), and an ambient temperature of 21 ± 2 °C (humidity approximately 40–60%). The animals had free access to filtered tap water and food (Nuvilab for rodents, Nuvital Nutrients S.A., Colombo, Paraná, Brazil). Acclimatization was performed for two weeks before the experimental period began. All procedures

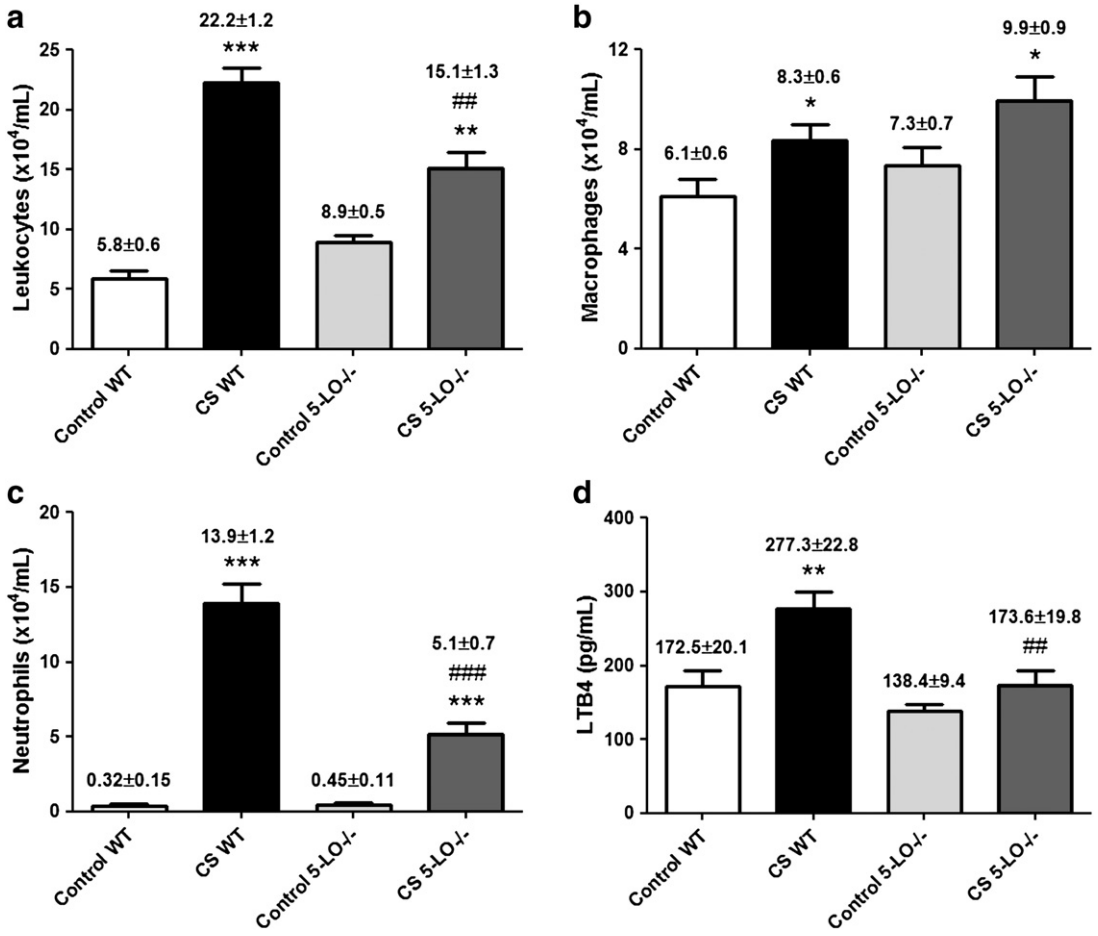

Fig. 1. Inflammatory markers. a) Total leukocytes in the bronchoalveolar lavage (BAL) fluid of mice exposed to cigarette smoke (CS) and Control groups. b) Macrophages in the BAL fluid of mice exposed to CS and Control groups. c) Neutrophils in the BAL of mice exposed to CS and Control groups. d) ELISA for LTB4 in the BAL fluid supernatant of mice exposed to CS and Control groups. 5-LO$^{-/-}$: 5-lipoxygenase knockout mice; WT: wild-type mice. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the respective Control groups. # # $p < 0.01$ and # # # $p < 0.001$ when compared with the WT CS group. Data are presented as the mean ± standard error ($n = 10$).

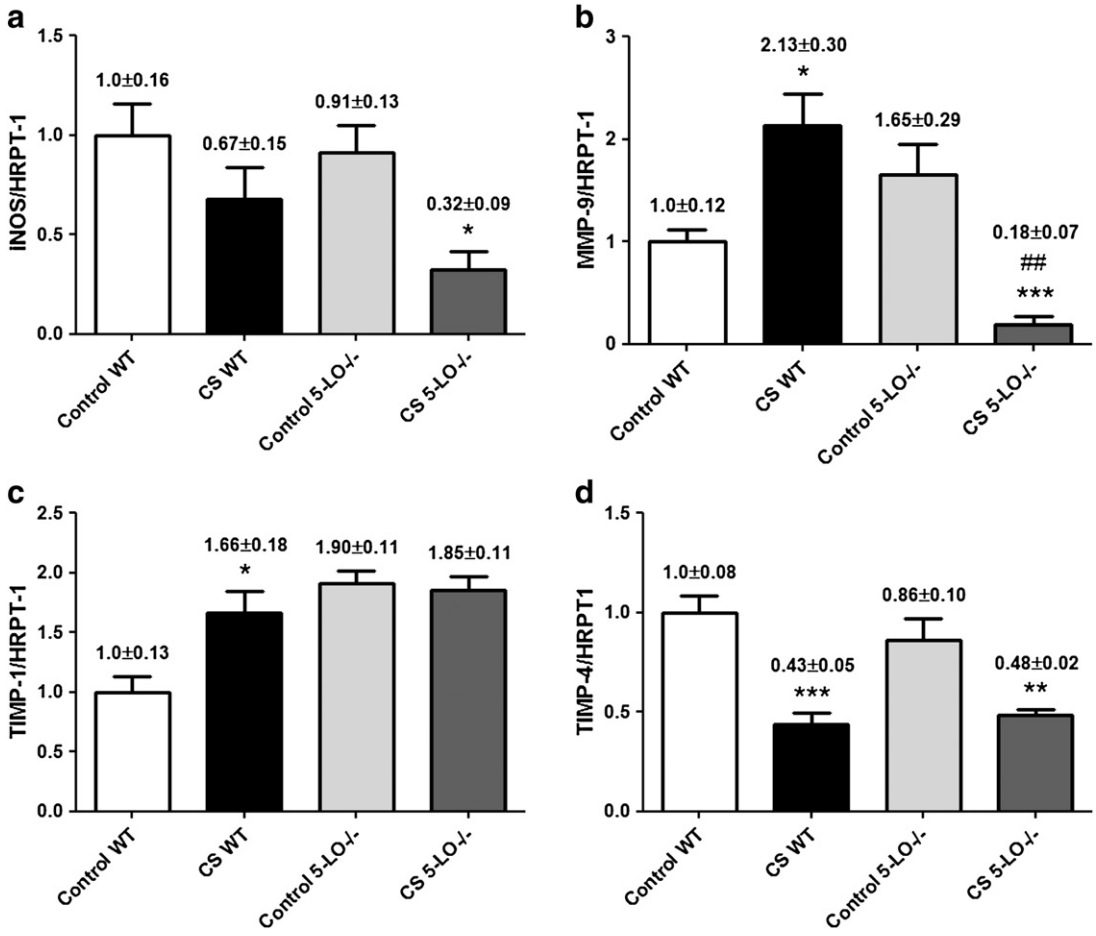

Fig. 2. qPCR of inflammatory and proteolytic markers. a) Expression of *inducible nitric oxide synthase* (*iNOS*). b) Expression of *matrix metalloproteinase-9* (*MMP-9*). c) Expression of *tissue inhibitor of matrix metalloproteinase 1* (*TIMP-1*). d) Expression of *tissue inhibitor of matrix metalloproteinase 4* (*TIMP-4*). All data were normalized to *hypoxanthine phosphoribosyl transferase* (*HPRT-1*) expression. 5-LO$^{-/-}$: 5-lipoxygenase knockout mice; WT: wild-type mice. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the respective Control groups. # # $p < 0.01$ and # # # $p < 0.001$ when compared with the WT CS group. Data are presented as fold induction ($n = 10$).

were performed in accordance with The Ethics Committee for Experimental Animal Use and Care (CEUA) of the Centro de Ciências da Saúde/Universidade do Federal do Rio de Janeiro. The CEUA follows guidelines from the Intramural Animal Care and Use (ACU) program of the National Institutes of Health (NIH).

2.2. Experimental design

Mice were exposed to CS for 60 days (CS groups) as described below. Mice exposed to ambient air (sham-smoked) were used as Controls ($n = 15$ each). To study the effects of CS, the mice were exposed to 12 commercial full-flavored Marlboro cigarettes (10 mg tar, 0.9 mg nicotine, and 10 mg monoxide) per day for 60 days using a smoking chamber, as described previously [15–20]. Briefly, the animals were placed in the inhalation chamber (40 cm long, 30 cm wide, and 25 cm high), which was placed inside an exhaust hood. In this condition, the exhaust fan was turned off. A cigarette was coupled to a plastic 60-mL syringe, and puffs of smoke were drawn into the syringe and then expelled into the inhalation chamber. One liter of smoke from each cigarette was aspirated with this syringe (20 puffs of 50 mL each), and each puff was immediately injected into the chamber. The animals were maintained in this smoke-filled air condition (±3%) for 6 min. Then, the cover of the inhalation chamber was removed, and the exhaust fan of the hood was turned on to evacuate the smoke. The smoke was evacuated within 1 min. This exposure to CS was repeated four times (4 × 6 min) with an exhaust interval of 1 min after each exposure. This procedure was repeated three times per day (morning, noon, and afternoon), which resulted in the mice being exposed to the smoke of twelve cigarettes over 72 min. Each cigarette produced ±300 mg/m^3 of total particulate matter in the chamber (measured by weighing material collected on Pallflex filters). The carboxyhemoglobin (COHb) levels were measured to confirm that the exposure was not toxic, as described previously [21].

2.3. Bronchoalveolar lavage and ROS analysis

The lung air spaces were washed three times with buffered saline solution (500 μL) for a final bronchoalveolar lavage (BAL) fluid volume of 1.2–1.5 mL ($n = 10$ per group). The collected BAL fluid was stored on ice. The total number of cells in the BAL fluid was determined using a Neubauer chamber. The differential cells were counted in cytospin sections stained with Giemsa. We used a nitroblue tetrazolium assay to determine the ROS production in the leukocytes from the BAL by a method adapted from Choi and colleagues [22].

2.4. Tissue processing, histology, and morphometry

After the BAL, the left lungs ($n = 10$ per group) were removed immediately, homogenized on ice with 10% (w/v) 0.1 M potassium phosphate buffer (pH 7.4) using a tissue homogenizer (Nova técnica homogenizer model NT136, Campinas, São Paulo, Brazil) and centrifuged at 800 ×g for 5 min. The supernatants were stored at −20 °C for biochemical analysis. The protein concentrations in the lung homogenate samples were determined by the Bradford method [23].

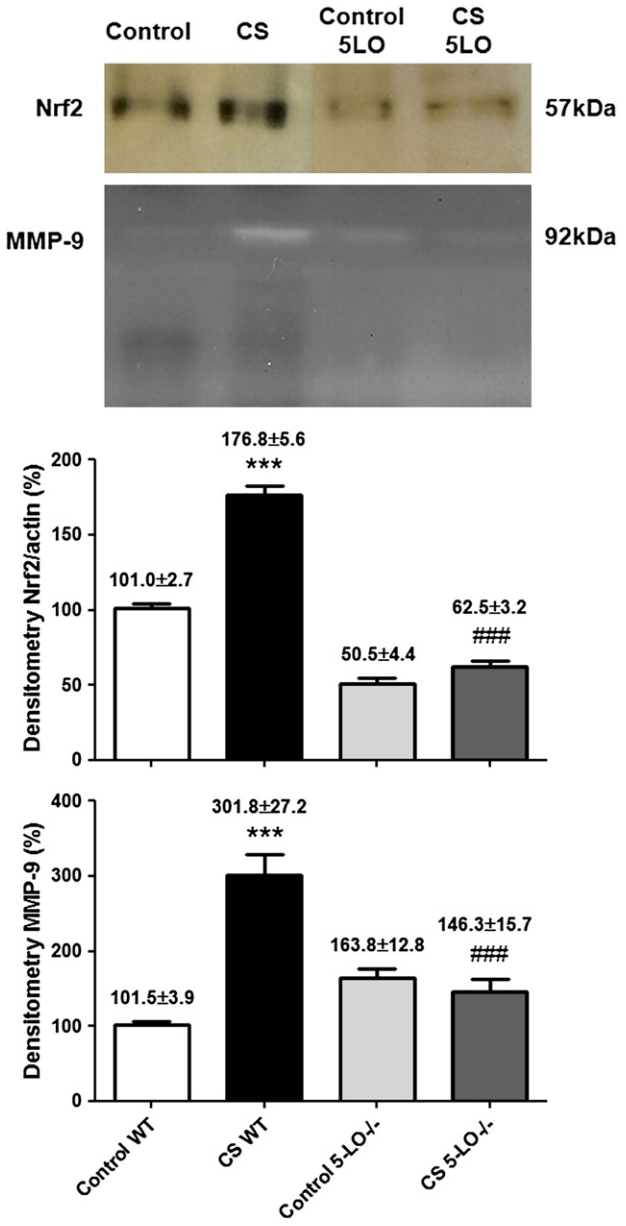

Fig. 3. Western blotting and zymography. Western blotting for Nrf2 from the nuclear extracts of lung tissue homogenates. Zymography was performed from lung tissue homogenates. Densitometry was calculated by three different samples and bands from western blotting were normalized using a loading control. 5-LO$^{-/-}$: 5-lipoxygenase knockout mice; WT: wild-type mice. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the respective Control groups. # # $p < 0.01$ and # # # $p < 0.001$ when compared with the WT CS group. Data are presented as the mean ± standard error ($n = 3$).

The right lungs were removed and fixed with 4% paraformaldehyde. The tissue was embedded in paraffin, and 5-μm-thick sections were stained with hematoxylin and eosin (H&E). The air-space enlargement was quantified by the mean linear intercept length of the distal air spaces (L_m) in 30 randomly chosen fields of tissue sections per group [24]. Fields with airways or blood vessels were not used for morphometry.

2.5. ELISA

Samples of BAL from the mice were used for the quantification of leukotriene B4 LTB4, which were measured using an ELISA kit (R&D, Minneapolis, MN, USA).

2.6. Thiobarbituric acid reactive substances

The malondialdehyde concentration was measured by the thiobarbituric acid reactive substances (TBARS) method, as described by Draper and Hadley [25].

2.7. Western blotting

Proteins from tissue homogenates were separated by PAGE and transferred to nitrocellulose membranes. After blocking, the membranes were incubated with Nrf2 goat anti-mouse antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were then incubated with horseradish peroxidase-antibody conjugate (1:2000). The membranes were then treated with chemiluminescent agents (luminol and enhancer, ECL Plus; Amersham Biosciences, Piscataway, NJ, USA) and exposed to light-sensitive film.

2.8. Zymography

Aliquots of lung homogenates were subjected to electrophoresis in an 8% acrylamide stacking gel—7% acrylamide separating gel containing 1 mg/mL gelatin in the presence of sodium dodecyl sulfate (SDS) under non-reducing conditions. MMP-2 and/or MMP-9 activities appeared as clear bands against the blue background. The molecular weights of the gelatinolytic bands were estimated using 30 μg of protein from a placental sample [26].

2.9. qPCR

Lung fragments were collected, snap frozen, and stored at −80 °C in RNAlater (QIAGEN, Valencia, CA, USA). Total RNA was isolated using the RNeasy tissue kit (Qiagen). After extraction, ~100 ng of RNA was used for cDNA synthesis using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA), according to the manufacturers protocol. The quantitative real-time PCR was performed on a 7500 real-time PCR System (Applied Biosystems), and the threshold cycle values were determined using RQ Study Software (Applied Biosystems). The expression of each gene was quantified and normalized to *hypoxanthine phosphoribosyl transferase* (*HPRT1*) expression, and the relative fold induction was calculated according to the formula $2^{-\Delta\Delta Ct}$ (Table 1).

2.10. Pulmonary function

A separate group of mice were sedated with diazepam (1 mg, i.p.), anesthetized with pentobarbital sodium (20 mg/kg BW, i.p.), tracheotomized, and a snugly fitting cannula (0.8 mm i.d.) was introduced into the trachea. The animals were then paralyzed with pancuronium bromide (0.1 mg/kg, i.v.) and mechanically ventilated with a constant flow ventilator (Samay VR15, Universidad de la Republica, Montevideo, Uruguay) with a respiratory frequency of 100 breaths/min, tidal volume of 0.2 mL, flow of 1 mL/s, and positive end-expiratory pressure of 2 cmH_2O. The anterior chest wall was then surgically removed. A pneumotachograph (15 mm i.d., length 4.2 cm, distance between side ports = 2.1 cm) was connected to the tracheal cannula for the measurement of airflow (V'). The lung volume (VT) was measured by flow signal integration. The pressure gradient across the pneumotachograph was determined using a Valydine MP45-2 differential pressure transducer (Engineering Corp., Northridge, CA, USA). The flow resistance of the equipment (Req), including the tracheal cannula, was constant up to flow rates of 26 mL/s and was equal to 0.12 $cmH_2O\ mL^{-1}\ s$. The equipment resistive pressure ($= R\text{eq} \cdot V'$) was subtracted from the pulmonary resistive pressure so that the results represent intrinsic values. The tracheal pressure was measured with a Validyne MP-45 differential pressure transducer (Engineering Corp. Northridge, CA, USA). All of the signals were conditioned and amplified in a Beckman type R Dynograph (Schiller Park, IL, USA). The flow

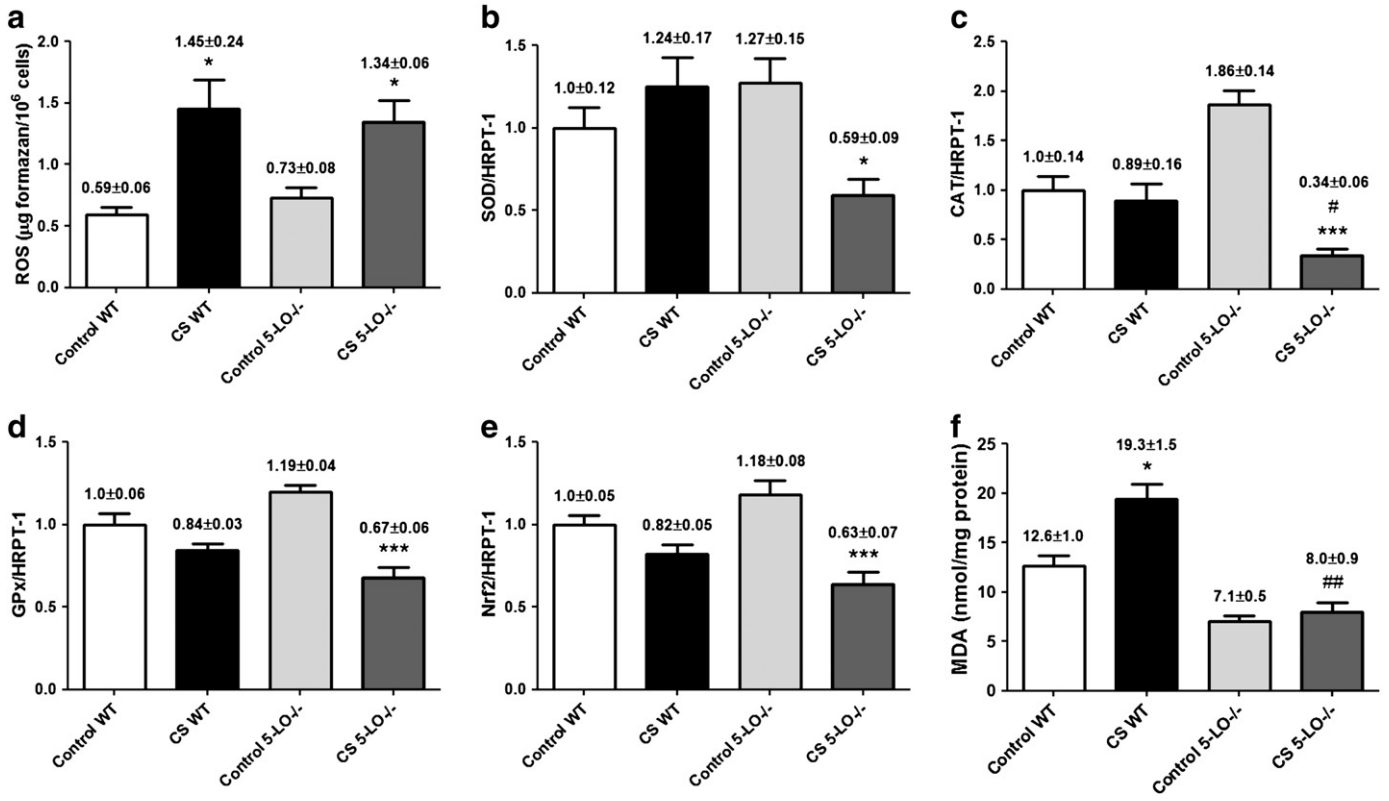

Fig. 4. Redox markers. a) Total reactive oxygen species (ROS) in BAL cells. b) Expression of *superoxide dismutase* (*SOD*) measured by real time PCR. c) Expression of *catalase* (*CAT*) measured by real time PCR. d) Expression of *glutathione peroxidase* (*GPx*) measured by real time PCR. e) Expression of *nuclear factor* (*erythroid-derived 2*)*-like 2* (*Nrf2*) measured by real time PCR. f) Levels of malondialdehyde in lung homogenates. The real-time PCR data were normalized to *hypoxanthine phosphoribosyl transferase* (*HRPT-1*) expression. 5-LO$^{-/-}$: 5-lipoxygenase knockout mice; WT: wild-type mice. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the respective Control groups. # # $p < 0.01$ and # # # $p < 0.001$ when compared with the WT CS group. Data are presented as the mean ± standard error ($n = 10$, a and f; $n = 5$, b, c, d, and e).

and pressure signals were passed through 8-pole Bessel low-pass filters (902 LPF, Frequency Devices, Haverhill, MA, USA) with the corner frequency set at 100 Hz, sampled at 200 Hz with a 12-bit analog-to-digital converter (DT2801A, Data Translation, Marlboro, MA, USA), and stored on a microcomputer. All data were collected using LABDAT software (RHT-InfoData Inc., Montreal, QC, Canada). The lung resistive (DPin,L) and viscoelastic/inhomogeneous (DPdi,L) pressures and the total resistive pressure drop (DPtot,L = DPin,L + DPdi,L) were measured by the end-inflation occlusion method [27]. Briefly, after the end-inspiratory occlusion, there is an initial rapid drop in the transpulmonary pressure (DPin,L) from the pre-occlusion value down to an inflection point (Pi), followed by a slow pressure decay (DPdi,L) until a plateau is reached. This plateau corresponds to the elastic recoil pressure of the lung (Pel). The DPin,L selectively reflects the airway resistance in normal animals and humans, and the DPdi,L reflects the stress relaxation or viscoelastic properties of the lung together with a small contribution of time constant inequalities at the peripheral airspaces.

2.11. Statistical analyses

All data are presented as the means ± standard error of the means, and after testing normality by the Kolmogorov–Smirnov test, the significance of differences was analyzed by one-way ANOVA followed by Tukey's post-hoc test, with $p < 0.05$. Additionally, we performed two-way ANOVA followed by Bonferroni's post-hoc test, with $p < 0.05$. The two-way ANOVA determined how genetic background influenced cigarette smoke exposure and if there was interaction between these two factors. The correlation was analyzed by Pearson with an alpha of 5%. GraphPad Prism 5 software was used for the statistical analysis (GraphPad Prism version 5.0, San Diego, CA, USA).

3. Results

3.1. Influence of 5-LO on inflammatory and proteolytic markers

The CS exposure resulted in a 279% increase of leukocyte influx to the lungs of the CS WT group compared with the Control WT group ($p < 0.001$). Although inflammatory cell counts were also increased in the CS 5-LO$^{-/-}$ group (69%; $p < 0.01$) compared with the 5-LO$^{-/-}$ group, these counts were reduced by 33% compared with the CS WT group ($p < 0.01$) (Fig. 1a). The macrophage and neutrophil counts were increased by 35% ($p < 0.05$) and 600% ($p < 0.001$) in the CS WT group compared with the Control WT group, respectively. The CS exposure resulted in less pronounced pulmonary inflammation in the CS 5-LO$^{-/-}$ group; the macrophage counts were similar, and the neutrophil counts were increased by approximately 35% compared with the CS WT group ($p < 0.001$) (Fig. 1b and c). Additionally, the LTB4 levels were only altered due to CS exposure in the CS WT group (35%, $p < 0.05$), whereas the CS 5-LO$^{-/-}$ group showed LTB4 values comparable with the 5-LO$^{-/-}$ group and were reduced by 37% when compared with the CS WT group ($p < 0.01$) (Fig. 1d).

Although *inducible nitric oxide synthase* (*iNOS*) expression was not altered in the CS WT group compared with the Control WT group, it was reduced by 33% in the CS 5-LO$^{-/-}$ group when compared with both the 5-LO$^{-/-}$ and CS WT groups ($p < 0.05$) (Fig. 2a). The *MMP-9* expression was increased by 100% in the CS WT group compared with the Control WT group ($p < 0.05$), whereas it was decreased by 75% ($p < 0.01$) and 83% ($p < 0.001$) in the CS 5-LO$^{-/-}$ group when compared with the 5-LO$^{-/-}$ and CS WT groups, respectively (Fig. 2b). The *tissue inhibitor of matrix metalloproteinase* (*TIMP*)*-1* expression was increased by 60% in the CS WT group compared with the Control WT group ($p < 0.05$) (Fig. 2c). The *TIMP-4* expression was decreased in

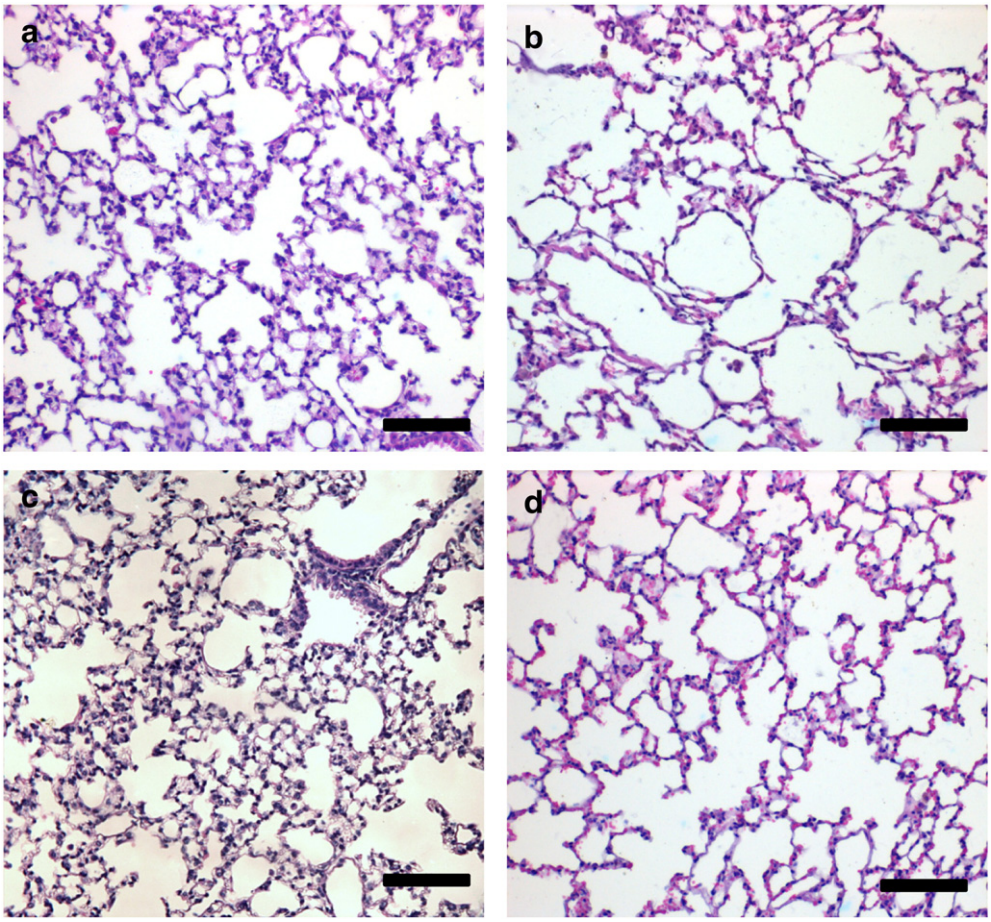

Fig. 5. Histology. a) Control WT group with normal alveolar septa and alveoli. b) CS WT group with enlarged alveoli. c) Control 5-LO$^{-/-}$ group with normal alveolar septa and alveoli. d) CS 5-LO$^{-/-}$ group with preserved alveolar septa and alveoli. All images are representative of 5 mice. Sections (5 μm) were stained with H&E. Figures were captured with a 20× objective lens. Scale bar = 100 μm.

both of the CS exposed groups compared with their respective Control groups ($p < 0.01$) (Fig. 2d).

The MMP-9 activity was increased by three-fold in the CS WT group compared to the Control WT group ($p < 0.001$). However, the MMP-9 activity was reduced by 48% in the CS 5-LO$^{-/-}$ group compared with the CS WT group ($p < 0.001$) (Fig. 3).

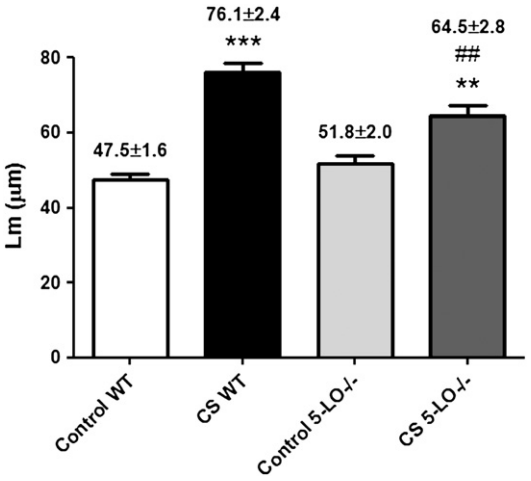

Fig. 6. Morphometry. Randomly selected fields (n = 30) were counted per mouse in 5 μm sections stained with H&E. Final magnification of images was 1000×. 5-LO$^{-/-}$: 5-lipoxygenase knockout mice; WT: wild-type mice. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the respective Control groups. # # $p < 0.01$ and # # # $p < 0.001$ compared with the WT CS group. Data are presented as the mean ± standard error (n = 5).

3.2. Influence of 5-LO on redox markers

The ROS levels were 145% ($p < 0.05$) and 83% ($p < 0.05$) in the CS-exposed groups compared with their respective Control groups (WT and 5-LO$^{-/-}$, respectively) (Fig. 4a). The CS exposure did not alter antioxidant enzyme expression in the WT animals. However, the expression of *superoxide dismutase* (*SOD*) (−50%), *catalase* (*CAT*) (−80%), and *glutathione peroxidase* (*GPx*) (−40%) was decreased in the CS 5-LO$^{-/-}$ group compared with the 5-LO$^{-/-}$ group (Fig. 4a-d). The nuclear factor (erythroid-derived 2)-like 2 (Nrf2) protein expression was reduced by 55% only in the CS 5-LO$^{-/-}$ group compared with 5-LO$^{-/-}$ group ($p < 0.01$) (Fig. 4e). The MDA levels were increased by 60% in the CS WT group compared with the Control WT group ($p < 0.05$), whereas, the MDA levels of the CS 5-LO$^{-/-}$ group were comparable to the 5-LO$^{-/-}$ group and were also reduced by 60% compared to the CS WT group ($p < 0.001$) (Fig. 4f). The Nrf2 protein expression was increased by 75% in the CS WT group compared with the Control WT group. However, the NRF-2 protein expression was reduced by 40% in the CS 5-LO$^{-/-}$ group compared to the CS WT group ($p < 0.001$) (Fig. 3).

3.3. Participation of 5-LO in lung histoarchitecture

The mice exposed to ambient air (Control group and 5-LO$^{-/-}$ group) showed a normal lung structure with intact alveoli and thin alveolar

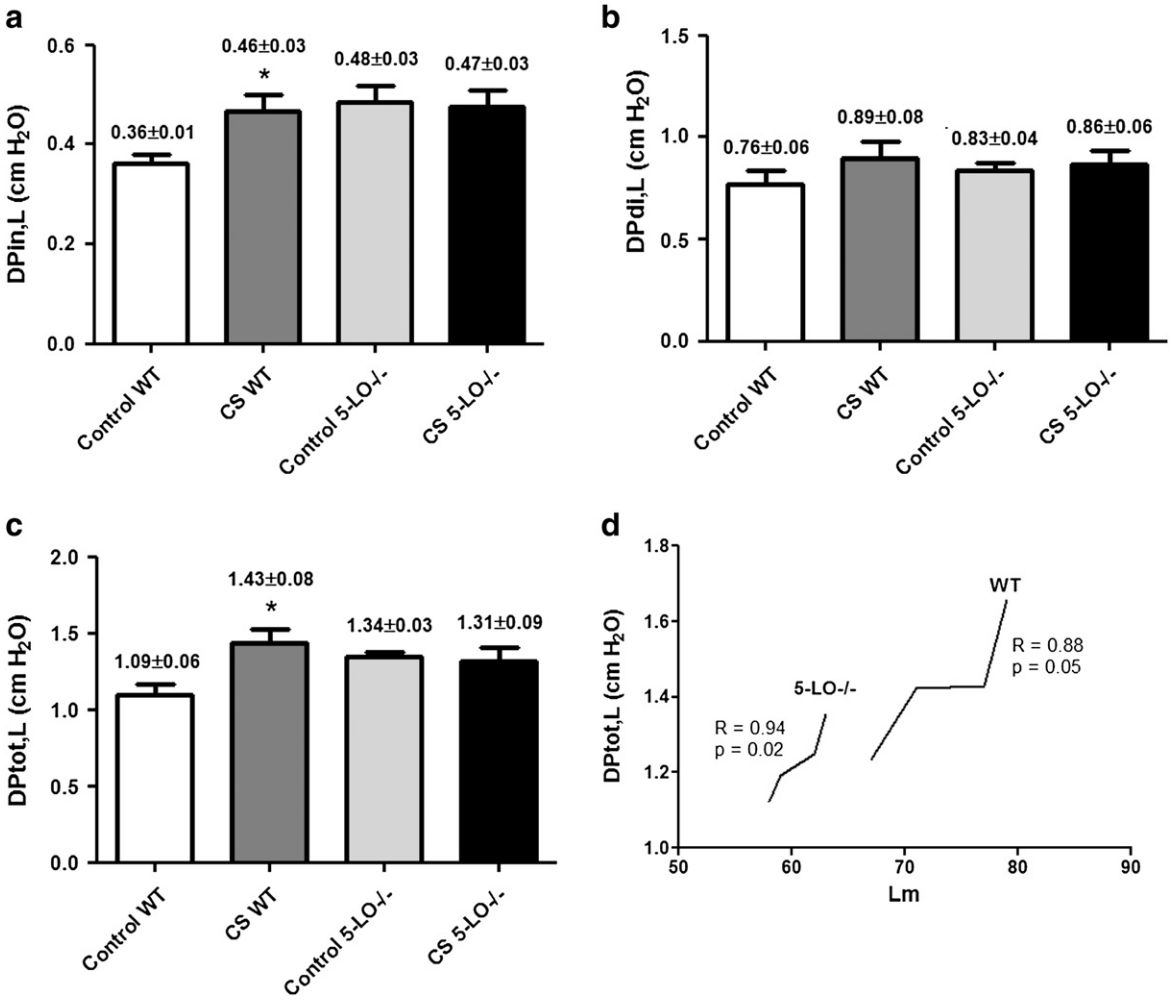

Fig. 7. Pulmonary function. Lung resistive (a — DPin,L) and viscoelastic/inhomogeneous (b — DPdi,L) pressures and total resistive pressure drop (c — DPtot,L = DPin,L + DPdi,L) were measured by the end-inflation occlusion method. d) Pearson correlation between DPtot,L and Lm parameters. 5-LO$^{-/-}$: 5-lipoxygenase knockout mice; WT: wild-type mice. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the respective Control groups. # # $p < 0.01$ and # # # $p < 0.001$ compared with the WT CS group. Data are presented as the mean ± standard error ($n = 5$). For correlation calculation: $n = 4$.

septa (Fig. 5a and c). The CS WT group showed a lung structure consistent with pulmonary emphysema, which included the enlargement of airspaces and the presence of inflammatory cells in the alveolar septa and alveoli (Fig. 5b and c).

The Lm was increased by 60% in the CS WT group compared with the Control WT group ($p < 0.001$). The Lm values were increased less in the CS 5-LO$^{-/-}$ group compared with the 5-LO$^{-/-}$ Control group (24%; $p < 0.01$). In addition, the Lm was decreased by 15% in the CS 5-LO$^{-/-}$ group compared with the WT CS group ($p < 0.05$) (Fig. 6).

3.4. Participation of 5-LO in lung function

The DPin,L (Fig. 7a) values were higher only in the CS WT group ($p < 0.05$) compared to the Control WT. The DPdi,L (Fig. 7b) values were similar among the groups. The Dptot,L (Fig. 7c) values were higher only in the WT CS group compared with the Control WT group ($p < 0.05$). To better understand the association between pulmonary function and the relation to alveolar destruction, a correlation between the Dptot,L and the Lm was performed (Fig. 7d). We observed that the Dptot,L and the Lm are correlated but that the correlation is higher in the CS 5-LO$^{-/-}$ group with a slight reduction in the CS WT group. This result demonstrates that the increase in the Lm and Dptot,L are parallel.

3.5. Genetic background vs. CS exposure

Table 2 shows the effects of genetic background versus cigarette smoke exposure. There was a modest but statistically significant interaction between CS exposure and genetic background on leukocytes and neutrophils, which are markers of inflammation. For proteolytic markers, there was a significant interaction between CS exposure and genetic background on MMP-9 but less so on TIMP-4. Regarding redox markers, there was a good interaction between CS exposure and genetic background on SOD and CAT and less over the GPx, Nrf2, and MDA. Morphometrically, there was a moderate but statistically significant interaction between CS exposure and genetic background on Lm, while there was a moderate but statistically significant interaction between CS exposure and genetic background on DPtot,L, a measure of pulmonary function.

4. Discussion

There are two classic ways to induce emphysema in mice: 1) by intratracheal or intranasal proteolytic enzymes or 2) by exposure to cigarette smoke [28–30]. In the present study, the involvement of 5-LO in cigarette smoke-induced lung emphysema in mice was analyzed. As previously described, lung emphysema was induced after 60 days of

Table 2
Two-way ANOVA — genetic background vs. exposure to cigarette smoke (CS).

Data	Interaction		Genetic background		Exposure to CS	
	%	P	%	P	%	P
Inflammatory markers						
Leukocytes ($\times 10^4$/mL)	14	0.001	2	No	71	0.001
Macrophages ($\times 10^4$/mL)	0	No	10	No	29	0.01
Neutrophils ($\times 10^4$/mL)	15	0.001	14	0.001	63	0.001
LTB4 (pg/mL)	7	No	31	0.01	32	0.01
iNOS/HRPT-1	3	No	8	No	36	0.01
Proteolytic markers						
MMP-9/HRPT-1	63	0.001	15	0.05	1	No
MMP-9 activity	45	0.001	8	0.05	35	0.001
TIMP-1/HRPT-1	19	0.05	44	0.01	13	No
TIMP-4/HRPT-1	3	No	0	No	73	0.001
Redox markers						
ROS (μg formazan/10^6 cells)	2	No	0	No	71	0.001
SOD/HRPT-1	43	0.01	7	No	9	No
CAT/HRPT-1	34	0.001	1	No	45	0.001
GPx/HRPT-1	17	0.01	0	No	58	0.001
Nrf2/HRPT-1	14	0.05	0	No	57	0.001
Nrf2 protein	10	0.001	68	0.001	19	0.001
MDA (nmol/mg protein)	8	0.05	69	0.001	14	0.01
Morphometry and pulmonary function						
Lm (μm)	10	0.01	2	No	70	0.001
DPin,L (cmH2O)	14	No	19	0.05	10	No
DPdi,L (cmH2O)	3	No	0	No	7	No
DPtot,L (cmH2O)	25	0.05	2	No	17	No

LTB4, leukotriene B4; iNOS, inducible nitric oxide synthase; HRPT-1, hypoxanthine phosphoribosyl transferase; MMP-9, matrix metalloproteinase 9; TIMP-1, tissue inhibitor of matrix metalloproteinase 1; TIMP-4, tissue inhibitor of matrix metalloproteinase 4; ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; Nrf2, nuclear factor (erythroid-derived 2)-like 2; MDA, malondialdehyde; Lm, mean linear intercept; DPin,L, lung resistive pressure; DPdi,L, lung viscoelastic/inhomogeneous pressure; DPtot,L total resistive pressure drop. Two-way ANOVA followed Bonferroni's post-test was performed with the mean ± SEM. n = 10 for inflammatory markers and *iNOS*, *MMP-9*, TIMP-*1*, and *TIMP-4*; n = 3 for western blotting and zymography; n = 5 for *SOD*, *CAT*, *GPx*, and *Nrf2*; n = 5 for morphometry and pulmonary function.

cigarette smoke exposure [15–20], and pulmonary inflammatory, physiological, and redox parameters were studied.

The involvement of 5-LO in the production of key inflammatory mediators has been well described [11,31,32]. However, this is the first study demonstrating that 5-LO deletion can protect mouse lungs from cigarette smoke-induced emphysema. Although emphysema is known to be a chronic inflammatory disease, there are reports of an increase in activated neutrophils in the sputum of patients with chronic obstructive pulmonary disease [33–35]. The results of the current study showed that the CS WT group showed a higher number of neutrophils in the BAL compared with the Control group; however, this recruitment of neutrophils was lower in the CS 5-LO$^{-/-}$ group when compared with the CS WT group. Neutrophils provide a strong line of defense for the immune system, however, these cells are sources of reactive metabolites of oxygen, lipid mediators, inflammatory cytokines, and enzymes that cause tissue damage [36]. In contrast to the recruitment of neutrophils, there was no difference in the number of macrophages between the groups exposed to CS despite the already expected elevation of the number of macrophages compared with the Control groups.

The LTB4 levels were elevated in the 5-LO$^{-/-}$ and WT mice exposed to CS; however, the LTB4 value was lower in the CS 5-LO$^{-/-}$ group. This result suggests that the reduction of LTB4 levels in the CS 5-LO$^{-/-}$ group is linked to the deficiency of 5-LO because this enzyme has a central role in the production of LTB4 [12,13,33]. High levels of leukotrienes have been documented in asthma and chronic inflammatory lung disease but with different pathophysiological features when compared with emphysema [37]. Based on these results, we can conclude that the inflammatory response to CS is lower in KO rather than WT mice, which suggests that inflammation caused by CS may be 5-LO-dependent, as previously suggested by Mobley and colleagues [31].

Although studies attribute a crucial role of macrophages in the pathogenesis of emphysema by their ability to produce matrix metalloproteinases (MMPs) [38], specifically MMP-12 [15,39,40], in this study, the number of macrophages in the CS 5-LO$^{-/-}$ mice was similar to the CS WT mice. qPCR analysis was performed for *MMP-12*, but there was no difference between the groups (data not shown). In addition, both the *MMP-9* expression and the MMP-9 activity were lower in the CS 5-LO$^{-/-}$ group compared with the 5-LO$^{-/-}$ group, which suggests that MMP-9, a gelatinase A enzyme, may play an important role on the proteolytic factor of CS-induced emphysema in the 129S2 strain of mice; whereas in C57BL/6 J mice, this key role is played by macrophage elastase (MMP-12) [40–42]. Studies have shown that smoking is related to the increase of several metalloproteinases, such as MMPs 1, 2, 8, 9, and 14, in human samples [35,43–45]. Because MMP-9 is an elastolytic enzyme associated with both macrophages and neutrophils, MMP-9 has the potential to cause emphysema, lung parenchyma destruction with potential alteration, and damage to the lung tissue and pulmonary function [44,46]. Additionally, MMP-9 levels and the MMP-9/TIMP-1 ratio have been shown to be elevated in the sputum of COPD patients [45]. In our study, there was a direct relationship between MMP-9 and TIMP-1 only in the CS WT group. Nonetheless, the increased expression of *TIMP-1* in both the 5-LO$^{-/-}$ and CS 5-LO$^{-/-}$ control groups suggests a protective factor against the establishment of emphysema. It has been shown that other MMPs might contribute to emphysema [47–50], but it is likely that neutrophil elastase plays a significant role. In particular, it has been demonstrated that the lesion of the matrix, which results in emphysema, is the end-result of crosstalk between MMP-12 from macrophages and neutrophil elastase from neutrophils, with the latter protease more responsible for the greater portion of the final proteolytic attack [51,52].

Nitric oxide (NO) is present in several biological processes, such as immune regulation, platelet aggregation, neurotransmission, and inflammation. NO is synthesized in cells from L-arginine by different isoforms of NOS, including constitutive NOS, endothelial NOS, and iNOS [53]. In the present study, *iNOS* expression was lower in the CS 5-LO$^{-/-}$ mice compared with the CS WT mice, although it has been shown that iNOS plays an important role in murine pulmonary emphysema [54–56]. Furthermore, our group has recently shown that iNOS activity plays a key role in the development of elastase-induced emphysema [57]. iNOS can be stimulated by various proinflammatory cytokines and expressed in several types of inflammatory cells, such as macrophages and neutrophils. Once induced, iNOS is able to produce NO for a long period of time, which characterizes its involvement in various pathological processes [11,53]. Thus, this result suggests that a reduction of the inflammatory response in the CS 5-LO$^{-/-}$ group is associated with the reduced expression of iNOS because this enzyme is directly involved in the generation of NO. The inactivation of antiproteases and the redox imbalance can occur by the action of ROS released from activated leukocytes [5]. In the present study, the release of ROS was elevated in both the CS WT and CS 5-LO$^{-/-}$ groups. The release of ROS and proteases is a classic event of activated leukocytes, which contributes to the inflammatory response and destruction of lung parenchyma [3,8,57].

Nrf2 is a key transcription factor that controls the antioxidant response and plays an important role in protecting the lung against oxidative diseases [58]. The present study shows a significant reduction in the expression of *Nrf2* with a concomitant reduction in the expression of the antioxidant enzymes *SOD*, *CAT* and *GPx* in the CS WT group compared with the Control WT group. SOD comprises the first line of defense against oxidative damage caused by superoxide anions. This event supports the reduction of CAT found in these animals because CAT is an important catalyst for H_2O_2 as well as GPx, and both have an action subsequent of SOD [7]. Briefly, chronic exposure to CS in WT animals is not associated with a decrease in enzymatic antioxidant defenses, as if it was not regulated by Nrf2. Concomitantly with this factor, the ROS

and MDA levels are increased. Interestingly, the CS 5-LO$^{-/-}$ group showed a reduction of antioxidant defenses and a parallel reduction of Nrf2 expression, and despite the increased ROS in this group, the MDA levels are similar to the Control group and reduced compared with the CS WT group.

In previous studies using the C57BL/6 J mouse strain, our group established that murine emphysema occurs due to a reduction of antioxidant enzymes and the concomitant increase in MMP-12 activity [15,17]. In the present study, the CS WT group (129S2 strain) showed a pattern of antagonistic pathogenesis of emphysema compared with the C57BL/6 J mice, i.e., there was no difference in antioxidant enzymes. In the CS 5-LO$^{-/-}$ group, despite showing a pattern of antioxidant enzymes similar to C57BL/6 J mice with emphysema, the mice were protected from the harmful effects of CS. This complex response difference suggests that the pattern of protection in the 5-LO$^{-/-}$ mice may depend on the type of MMPs/TIMPs secreted and not exactly the type of response to oxidative stress. Furthermore, it was observed that CS causes a dual response in the pathogenesis of emphysema in C57BL/6 J mice: via MMPs and oxidative stress. As in 129S2 mice, the main component of emphysema pathogenesis is via MMPs.

The present murine model of CS-induced emphysema was able to promote morphological and morphometric alterations in the lungs of mice. However, the changes in the lung architecture in the CS 5-LO$^{-/-}$ were less evident compared with the CS WT group. This evidence is based on Lm analysis, a reliable parameter for the quantification/characterization of pulmonary emphysema [24]. However, this change was not enough to promote significant alterations in lung function. However, compared with the Control mice, we observed a correlation between the WT and 5-LO$^{-/-}$ mice exposed to CS regarding histology and pulmonary function; the greater the Lm the higher the total pulmonary pressure delta. These data suggest that alterations in lung morphology affect pulmonary function as well.

CS had a greater impact compared with genetic background on influencing the alterations observed in inflammation, redox markers, proteolytic, and morphofunctional parameters. However, overall there was a reasonable interaction between genetic background and CS exposure, particularly regarding proteases and antiproteases. In conclusion, we show for the first time that 5-LO deficiency protects 129S2 mice against emphysema caused by CS. We suggest that the main mechanism of pathogenesis in this model is due to the imbalance between proteases and anti-proteases, particularly the association between MMP-9 and TIMP-1. Although 129S2 mice might not be the best model for studying murine emphysema, these mice still share some main peculiarities with more popular strains (A/J and C57BL/6 J), such as Lm, inflammatory markers, and also the relationship between MMP-9 and TIMP-1, which has been well established in humans with COPD. Accordingly, we suggest that 5-LO may be a potential therapeutic target for COPD in humans.

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