

Impact of lung remodelling on respiratory mechanics in a model of severe allergic inflammation

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Abstract

We developed a model of severe allergic inflammation and investigated the impact of airway and lung parenchyma remodelling on *in vivo* and *in vitro* respiratory mechanics. BALB/c mice were sensitized and challenged with ovalbumin in severe allergic inflammation (SA) group. The control group (C) received saline using the same protocol. Light and electron microscopy showed eosinophil and neutrophil infiltration and fibrosis in airway and lung parenchyma, mucus gland hyperplasia, and airway smooth muscle hypertrophy and hyperplasia in SA group. These morphological changes led to *in vivo* (resistive and viscoelastic pressures, and static elastance) and *in vitro* (tissue elastance and resistance) lung mechanical alterations. Airway responsiveness to methacholine was markedly enhanced in SA as compared with C group. Additionally, IL-4, IL-5, and IL-13 levels in the bronchoalveolar lavage fluid were higher in SA group. In conclusion, this model of severe allergic lung inflammation enabled us to directly assess the role of airway and lung parenchyma inflammation and remodelling on respiratory mechanics.

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1. Introduction

The pathology of severe asthma is characterized by a number of structural changes, including epithelial detachment and thickening of the reticular basement membrane (Kay, 1996), mucus gland hyperplasia (Hamid, 2003), subepithelial fibrosis (Hamid, 2003), elastosis and fragmentation of the elastic fibres (Mauad et al., 1999), inflammatory cell infiltration (de Magalhaes Simões et al., 2005), bronchial smooth muscle hypertrophy/hyperplasia

(Seow et al., 1998), and vascular changes (Hamid, 2003). Physiologic and pathologic data suggest that these alterations extend beyond the central airways to the distal airways and the lung parenchyma (Carroll et al., 1997; de Magalhaes Simões et al., 2005), making it difficult to obtain tissue samples (Wenzel, 2005). Thus, most of these pathological changes in asthmatic airways have only been investigated postmortem (Kay, 1996).

Murine models of allergic bronchopulmonary inflammation proved to be useful to examine the structural remodelling events, basic mechanisms of allergic inflammation, and the underlying immunologic response (Wagers et al., 2002, 2007; Kumar and Foster, 2002). There are few descriptions of rodent models of severe allergic inflammation (Mukaiyama et al., 2004; Beavitt et al., 2005) and they focused mainly on inflammatory and airway responsiveness processes. Recently, Ochkur et al. developed a double transgenic mouse model with several pulmonary patholo-

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gies representative of severe human asthma. Even though these models described histological and functional data that resembled severe human asthma (Beavitt et al., 2005; Ochkur et al., 2007), none of them studied the consequences of airway and lung parenchyma histological changes on *in vivo* and *in vitro* lung mechanics, not did they correlate the cellular and immune responses linked to the pathologies.

Thus, in the present study we aimed to develop a mouse model of allergic inflammation with histological features of severe human asthma, such as damage of airway epithelium, subepithelial fibrosis, airway smooth muscle hypertrophy and hyperplasia, eosinophil and neutrophil infiltration, and mucus gland hyperplasia. We investigated the impact of these pathological changes and cellular and immune modifications on *in vivo* and *in vitro* lung mechanics and methacholine-induced hyperresponsiveness.

2. Materials and methods

2.1. Animal preparation

A total of 62 BALB/c mice (20–25 g) were used. In the severe allergic lung inflammation group (SA) ($n = 8$), mice were immunized, using an adjuvant-free protocol, by the intraperitoneal injection of 10 μg sterile ovalbumin (OVA) in 0.1 ml saline on each of seven alternate days. Forty days after the beginning of sensitization, 40 μg OVA in 20 μl warm sterile saline were intratracheally instilled. This procedure was performed three times with a 3-day interval between them. The control group (C) received saline using the same protocol ($n = 8$). These doses were based on a series of pilot experiments to provide a model of severe allergic lung inflammation with several pulmonary pathologies representative of severe human asthma.

2.2. Measurement of pulmonary mechanics

Twenty-four hours after the last challenge, the animals were sedated (diazepam 1 mg i.p.), anesthetized [pentobarbital sodium (20 mg kg body weight⁻¹ i.p.)], and a snugly fitting cannula (0.8 mm i.d.) was introduced into the trachea. Mechanical ventilation (model 683, Harvard Apparatus, Southnatick, MA, USA) with a frequency of 100 breaths min⁻¹, a tidal volume of 0.2 ml, and a positive end-expiratory pressure (PEEP) of 2 cmH₂O was applied. The anterior chest wall was surgically removed.

A pneumotachograph was connected to the tracheal cannula for the measurements of airflow (V') and changes in lung volume (V_T). The pressure gradient across the pneumotachograph was determined by means of a Validyne MP45-2 differential pressure transducer (Engineering Corp, Northridge, CA, USA). The flow resistance of the equipment (Req), tracheal cannula included, was constant up to flow rates of 26 ml s⁻¹, and amounted to 0.12 cmH₂O ml⁻¹ s. Equipment resistive pressure ($= \text{Req}V'$) was subtracted from pulmonary resistive pressure so that the results represent intrinsic values. Tracheal pressure (Ptr) was measured with a differential pressure transducer (Engineering Corp, Northridge, CA, USA). All signals were conditioned

and amplified in a Beckman type R Dynograph (Schiller Park, IL, USA). Flow and pressure signals were also passed through eight-pole Bessel filters (902LPF, Frequency Devices, Haverhill, MA, USA) with the corner frequency set at 100 Hz, sampled at 200 Hz with a 12-bit analogue-to-digital converter (DT2801A, Data Translation, Marlboro, MA, USA), and stored on a micro-computer. All data were collected using LABDAT software (RHT-InfoData Inc., Montreal, Quebec, Canada).

Muscle relaxation was achieved with vecuronium bromide (0.005 mg kg body weight⁻¹ i.v.), and a constant flow ventilator provided artificial ventilation (Samay VR15, Universidad de la Republica, Montevideo, Uruguay). Special care was taken to keep tidal volume ($V_T = 0.2$ ml) and flow ($V' = 1$ ml s⁻¹) constant in all animals in order to avoid the effects of different flows, volumes, and inspiratory duration on the measured variables.

Pulmonary mechanics were measured by the end-inflation occlusion method (Bates et al., 1988). In an open chest preparation, Ptr reflects transpulmonary pressure (PL). Pulmonary resistive (ΔP_1), viscoelastic/inhomogeneous (ΔP_2) pressures, $\Delta P_{\text{tot}} (= \Delta P_1 + \Delta P_2)$, and static elastance (Est) were determined. Pulmonary mechanics measurements were performed 10 times in each animal.

Data analysis was performed with ANADAT software (RHT-InfoData Inc., Montreal, Quebec, Canada).

2.3. Measurement of tissue mechanics

Heparine (1000 IU) was intravenously injected immediately after the determination of respiratory mechanics. The trachea was clamped 10 min later at end-expiration, and the abdominal aorta and vena cava were sectioned, yielding a massive haemorrhage that quickly killed the animals. The lungs were removed *en bloc*, and placed in a modified Krebs–Henseleith (K–H) solution (mM: 118.4 NaCl, 4.7 KCl, 1.2 K₃PO₄, 25 NaHCO₃, 2.5 CaCl₂·H₂O, 0.6 MgSO₄·H₂O, and 11.1 glucose] at pH 7.40 and 6 °C (Rocco et al., 2001; Xisto et al., 2005). Strips (2 mm × 2 mm × 10 mm) were cut from the periphery of the left lung and suspended vertically in a K–H organ bath maintained at 37 °C, continuously bubbled with a mixture of 95% O₂–5% CO₂ (Lopez-Aguilar and Romero, 1998; Rocco et al., 2001; Xisto et al., 2005). Metal clips made of 0.5 mm-thick music wire were glued to both ends of the tissue strip with cyanoacrylate. One clip was attached to a force transducer (FT03, Grass Instruments Co., Quincy, MA, USA), whereas the other one was fastened to a vertical rod. This fibreglass stick was connected to the cone of a woofer, which was driven by the amplified sinusoidal signal of a waveform generator (3312A Function Generator, Hewlett Packard, Beaverton, OR, USA). A sidearm of the rod was linked to a second force transducer (FT03, Grass Instruments Co., Quincy, MA, USA) by means of a silver spring of known Young's modulus, thus allowing the measurement of displacement. Length and force output signals were conditioned (Gould 5900 Signal Conditioner Frame, Gould Inc., Valley View, OH, USA), fed through eight-pole Bessel filters (902LPF Frequency Devices, Haverhill, MA, USA), analogue-to-digital converted (DT2801A, Data Translation Inc., Marlboro, MA, USA), and stored on a computer. All data were collected using LABDAT

software (RHT-InfoData Inc., Montreal, Quebec, Canada). The frequency response of the system was dynamically studied by using calibrated silver springs with different elastic Young's modulus. The hysteresivity of the system was independent of frequency and had a value <0.003 (Lopez-Aguilar and Romero, 1998; Rocco et al., 2001; Xisto et al., 2005).

After preconditioning each parenchymal strips tissue resistance (R), elastance (E), and hysteresivity (η) were calculated from the oscillatory recordings (Fredberg and Stamenovic, 1989).

2.4. Measurement of airway responsiveness

In other experimental groups, 16 animals (C and SA groups, $n=8$ /group) were submitted to the aforementioned protocols to measure airway responsiveness. One dose of saline following by increasing doses of methacholine (Sigma Chemical Co., Saint Louis, MI, USA) (3, 10, 30, 100, 300, 1000, 3000, and 10,000 $\mu\text{g/kg}$) were administered via a systatic catheter tied into the jugular vein and data were stored at 30 s, 1, 3, 5 and 7 min after agonist injection. Airflow and pressure were measured directly after achieving the maximal increase in Ptr that followed each intravenous infusion of increasing concentrations of methacholine (Arantes-Costa et al., 2002; Xisto et al., 2005). Respiratory system resistance was obtained using the equation of motion of the respiratory system: $\text{Ptr}(t) = EV(t) + RV'(t)$ where t is time.

2.5. Lung morphometric analysis

Morphometric analysis was performed in excised lungs at end-expiration. Immediately after the removal of the lungs *en bloc*, the right lung was quick-frozen by immersion in liquid nitrogen, and fixed with Carnoy's solution (Nagase et al., 1992). After fixation, the tissue was embedded in paraffin and 4- μm -thick slices were cut. The slides were stained with haematoxylin–eosin.

Morphometric analysis of lung architecture was performed with an integrating eyepiece with a coherent system made of a 100-point grid consisting of 50 lines of known length, coupled to a conventional light microscope (Axioplan, Zeiss, Oberkochen, Germany). The volume fraction of collapsed and normal pulmonary areas were determined by the point-counting technique (Weibel, 1990), made at a magnification of $200\times$ across 10 random non-coincident microscopic fields. Total cellularity and polymorphonuclear cells and pulmonary tissue were evaluated at $1000\times$ magnification. Points falling on cells were counted, and divided by the total number of points falling on tissue area in each microscopic field.

The areas of airway lumen, smooth muscle layer and airway epithelium were computed by counting the points falling on the airway lumen and those falling on airway smooth muscle and epithelium, respectively. The perimeter of the airways was estimated by counting the intercepts of the lines of the integrating eyepiece with the epithelial basal membrane. The areas of smooth muscle and airway epithelium were corrected in terms of airway perimeter by dividing their values by the number of inter-

cepts of the line system with the epithelial basal membrane of the corresponding airway. Because the number of intercepts (NI) of the lines with the epithelial basal membrane is proportional to the airway perimeter, and the number of points (NP) falling on airway lumen is proportional to airway area, the magnitude of bronchoconstriction [contraction index (CI)] was computed by the relationship: $\text{CI} = \text{NI}/\sqrt{\text{NP}}$ (Nagase et al., 1992).

Collagen (Picrosirius-polarization method) (Montes, 1996) and elastic fibres (Weigert's resorcin fuchsin method with oxidation) (Weigert, 1898) were quantified in the alveolar septa and in the airways. The alveolar septa quantification was carried out with the aid of a digital analysis system and specific software (Image-Pro® Plus 4.1 for Windows® (Media Cybernetics-Silver Spring, MD, USA) under $200\times$ magnification. The images were generated by a microscope (Axioplan, Zeiss, Oberkochen, Germany) connected to a camera (Sony Trinitron CCD, Sony, Tokyo, Japan), fed into a computer through a frame grabber (Oculus TCX, Coreco Inc., St Laurent, PQ, Canada) for off-line processing. The area occupied by fibres was determined by digital densitometric recognition. To avoid any bias due to septal oedema or alveolar collapse the areas occupied by the elastic and collagen fibres, measured in each alveolar septum were divided by the length of each studied septum. The results were expressed as the amount of elastic and collagen fibres per unit of septum length ($\mu\text{m}^2/\mu\text{m}$). The airways measurements were made with the aid of image analyses in a $200\times$ magnification, the whole airway wall was analyzed, two airways per animal. Collagen and elastic fibres content was quantified in the whole circumference of the two largest, transversally cut airways present in the sections. Transversally cut airways are defined when the short/long diameter ratio is larger than 0.6. Results were expressed as the area of collagen or elastic fibres divided by the perimeter of the basement membrane ($\mu\text{m}^2/\mu\text{m}$).

Five- μm thick slides of lung tissue were stained with Luna staining for eosinophils identification (Ying et al., 2002). By conventional morphometry, we assessed the density of eosinophils in airway and vessel walls. Using a 100-point grid with a known area (10,000 μm^2 at a $1000\times$ magnification) attached to the ocular of the microscope; we counted the number of points hitting the outer area of the airway wall (located between the external limit of smooth muscle layer and the adventitia) and the number of points hitting the adventitia of vessel walls. The tissue area in each microscopic field was calculated according to the number of points hitting the tissue, as a proportion of the total grid area. We then counted the number of eosinophils within that tissue area. Eosinophil density was determined as the number of eosinophils in each field divided by tissue area. Measurements were expressed as cells/ mm^2 (Weibel, 1990). Counting was performed in four airways and four vessels (12–18 fields) in each animal, at a magnification of $1000\times$.

2.6. Transmission electron microscopy

To obtain a stratified random sample, three samples of 2 mm $\times$ 2 mm were cut from different segments of the right lung, and then fixed with glutaraldehyde 2.5% and phosphate buffer 0.1 M (pH 7.4) for 60 min at -4°C . Ultrathin sections from

Table 1
Morphometric parameters

Groups	Normal area (%)	Alveolar collapse (%)	Contraction index	Total cellularity (%)	PMN (%)
C	94.3 ± 1.1	5.6 ± 1.0	1.41 ± 0.06	27.76 ± 0.53	8.22 ± 0.62
SA	62.1 ± 4.3*	37.2 ± 4.2*	1.73 ± 0.18*	31.29 ± 0.56*	25.06 ± 0.69*

Values are means (±S.E.M.) of eight animals in each group. In severe allergic inflammation group (SA) mice were sensitized with ovalbumin and exposed to repeated challenges with intratracheal instillation of ovalbumin. The control group (C) received 0.1 ml of saline under the same protocol. PMN: polymorphonuclear. Data were gathered from 10 random, non-coincident fields per mouse.

* Significantly different from C group ($p < 0.05$).

selected areas were examined and micrographed in a JEOL electron microscope (JSM-6100F; Tokyo, Japan). Submicroscopic analysis of lung tissue showed that the extension and distribution of the parenchymal alterations were inhomogeneous along the bronchiole and alveolar tissue (alveolar ducts and alveoli). Thus, electron micrographs representative of the lung specimen (C and SA groups) were enlarged to a convenient size to visualize the following parameters: (a) collapsed and normal pulmonary areas (Katzenstein, 1985; Burkhardt, 1989); (b) hyperinflated structures with a morphology distinct from that of alveoli and wider than 120 μm ; (c) airways: epithelial decapitation, basement membrane, collagen deposition, mucus gland, smooth muscle (Jeffery et al., 1992); and (d) eosinophils (Jeffery et al., 1992), neutrophils and lymphocytes.

The severity of the various submicroscopic processes was rated semi-quantitatively according to the amount and pathology by the following submicroscopic score:

- 0: absence of alterations,
- +: alterations in 1–25% of the tissue examined,
- ++: alterations in 26–50% of the tissue examined,
- +++: alterations in 51–75% of the tissue examined, and
- ++++: alterations in 76–100% of the tissue examined.

The semi-quantitative scores of ultrastructural lesions represent the average of extension and intensity of lesions present in all submicroscopic sections (three or four) examined, which in turn, represent the more compromised areas identified on electron microscopic examination.

2.7. Evaluation of bronchoalveolar lavage fluid (BALF)

Bronchoalveolar lavage fluid was obtained in parallel group of animals (C and SA groups, $n = 10/\text{group}$). The animals were killed in a CO_2 chamber and BALF collection was performed by infusion and extraction of 1 ml of 37 °C sterile, pyrogen-free saline (0.9% NaCl) via the tracheal cannula. This was repeated three times. Total leukocyte counts in the BALF were performed in Neubauer chambers under optical microscopy after diluting the samples in Türk solution (2% acetic acid). Differential leukocyte counts were performed in cytospin smears stained by the May–Grünwald–Giemsa method. Levels of IL-4, IL-5, and IL-13 in the cell-free BALF were evaluated by ELISA in accordance with the manufacturer's instructions (Duo Set, R&D Systems, Minneapolis, USA).

2.8. Statistical analysis

SigmaStat 2.0 statistical software package (Jandel Corporation, San Raphael, CA, USA) was used. Differences between the two groups were assessed by Student's t -test. Correlation between mechanical and histological data was determined by Spearman correlation test. A p value < 0.05 was considered significant.

3. Results

The fraction of alveolar collapse and contraction index increased in SA group in relation to C group (Table 1). Tissue cellularity was higher in SA group in comparison to C group (Table 1, Fig. 1). Luna stained slides showed an increase in the number of eosinophils in SA group (CTRL = 228 ± 32 and SA = 645 ± 31 , $p < 0.001$) (Fig. 1).

The total number of cells in the BALF was significantly higher in SA group than in C group, mainly eosinophils. SA group exhibited marked elevations in the amounts of IL-4, IL-5, and IL-13 in the BALF compared to C mice (Table 2).

Collagen fibre content in the alveolar septa (mean $\pm$ S.E.M.) (C = $0.03 \pm 0.001 \mu\text{m}^2/\mu\text{m}$, SA = $0.24 \pm 0.02 \mu\text{m}^2/\mu\text{m}$) and airways (C = $6.55 \pm 0.31 \mu\text{m}^2/\mu\text{m}$, SA = $15.49 \pm 1.01 \mu\text{m}^2/\mu\text{m}$) was greater in SA than in C group. Elastic fibre content in alveolar septa (C = $0.35 \pm 0.01 \mu\text{m}^2/\mu\text{m}$, SA = $0.36 \pm 0.02 \mu\text{m}^2/\mu\text{m}$) and airways (C = $1.09 \pm 0.02 \mu\text{m}^2/\mu\text{m}$, SA = $1.10 \pm 0.03 \mu\text{m}^2/\mu\text{m}$) were similar in C and SA groups.

Electron microscopy of SA group showed a prominent mucosal oedema which in turn led to a detachment between the epithelium and basement membrane (Fig. 2D). Important dam-

Table 2
Bronchoalveolar lavage cell count and inflammatory cytokines

	C	SA
Total cells (10^6 ml^{-1})	1.88 ± 0.16	10.57 ± 0.50*
Eosinophils (10^6 ml^{-1})	0.6 ± 0.7	6.80 ± 0.37*
Neutrophils (10^6 ml^{-1})	0.38 ± 0.17	1.03 ± 0.30*
Monocytes (10^6 ml^{-1})	0.90 ± 0.03	2.73 ± 0.31*
IL-4 (pg ml^{-1})	38.67 ± 8.72	89.89 ± 11.87*
IL-5 (pg ml^{-1})	92.73 ± 7.10	160.21 ± 15.83*
IL-13 (pg ml^{-1})	882.97 ± 40.95	2378.88 ± 55.72*

Values are means (±S.E.M.) of 10 animals in each group. In SA group mice were sensitized with ovalbumin and exposed to repeated challenges with intratracheal instillation of ovalbumin. The control group (C) received saline under the same protocol.

* Significantly different from C group ($p < 0.05$).

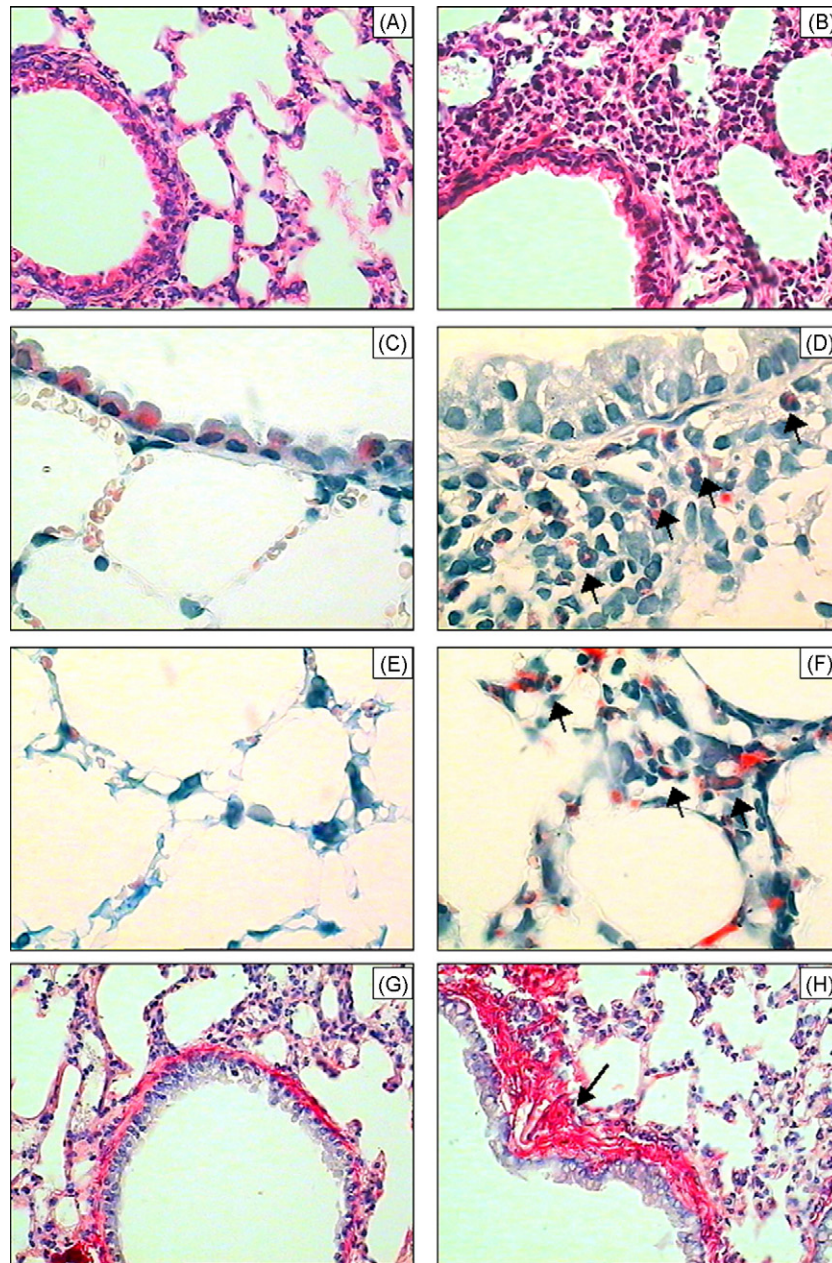


Fig. 1. Representative photomicrographs of airways and lung parenchyma in control (left panels) and severe allergic inflammation (right panels) groups. (Panels A and B) Lungs were stained with hematoxylin–eosin. (Panels C–F) Luna staining was used. (Panels D and F) Eosinophilic airway (D) and parenchyma (F) inflammation was observed (arrows). (Panels G and H) Picrosirius-polarization method was used. Note the increased deposition of collagen fibre in SA group (arrow). In the SA group, mice were sensitized with ovalbumin and exposed to repeated challenges with intratracheal instillation of ovalbumin. Scale bar in A, B, G, and H = 25 μ m. Scale bar in C, D, E, and F = 10 μ m.

age and shedding of the airway surface epithelium was present (Fig. 2C). Because of airway epithelial damage, smooth muscle was directly in contact with the bronchiolar lumen (Fig. 2C). The surface epithelium basement membrane was denuded (Fig. 2C). Mucous cells were disarranged and ciliated cells were damaged (Fig. 2D). A marked cellular infiltration of the airway wall included lympho-mononuclear cells and eosinophils. In some animals the neutrophils were in contact with the smooth muscle bundles (Fig. 2B and C, Table 3). The alveolar space showed preserved type I and type II pneumocytes overlying the apposed basement membrane (Fig. 2E).

There was no statistically significant difference in flow and volume between the groups. ΔP_1 , ΔP_2 , ΔP_{tot} , Est, E , and R were higher in SA than in the C groups. Hysteresivity was similar in all groups (Table 4). SA mice exhibited higher airway hyperresponsiveness compared with control (Fig. 3).

Considering C and SA groups together, static elastance and viscoelastic/inhomogeneous pressure were correlated with the fraction of area of alveolar collapse and total cellularity in the alveolar septa. ΔP_1 was correlated with the contraction index. Dynamic elastance and tissue resistance were correlated to the amount of collagen fibre (Table 5).

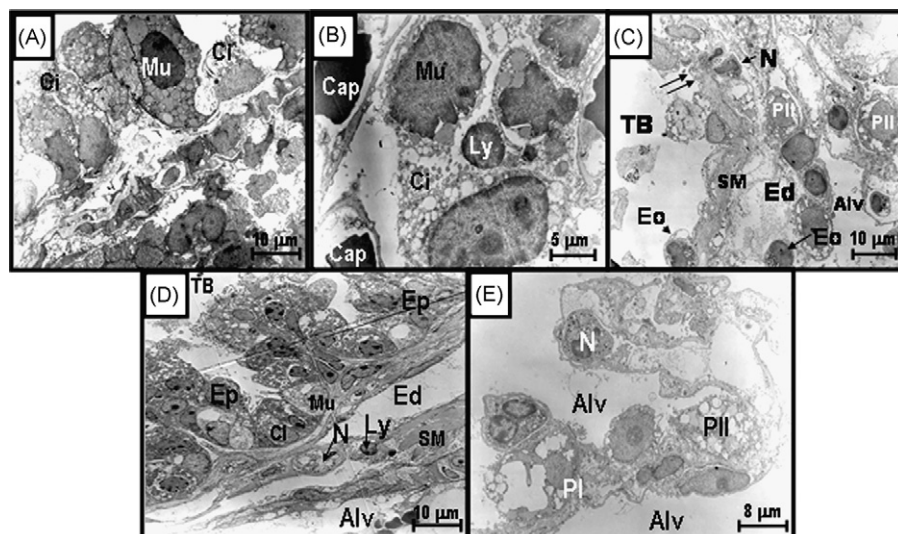


Fig. 2. Electron microscopy of terminal bronchiole and alveolar space in C (A) and SA (B–E) groups. (Panels A and B) Note mucus (Mu) and ciliated (Ci) cells. Ly: lymphocytes, Cap: capillary. (Panel C) Epithelium (Ep) basement membrane (arrows) is damaged. Eo: eosinophil. Ed: oedema. Alv: alveolar space. Neutrophils (N) are in contact with the smooth muscle (SM) bundles. In addition, the epithelium basement membrane is denuded. (Panel D) The epithelium basement membrane is evident with details of oedema (arrows). (Panel E) Type I pneumocytes (PI) and type II pneumocytes (PII) were normal. The photos are representative of electron microscopy changes observed in six specimens from C and SA groups.

Table 3
Ultrastructural and histological changes in the murine model of severe allergic lung inflammation

Features	Severe allergic lung inflammation murine model
Shedding and damage of airway epithelium	+++
Airway oedema	+++
Intra-epithelial neutrophils	++
Intra-epithelial lymphocytes	++
Eosinophilic infiltration	++++
Bronchial vessel congestion	+++
Subepithelial fibrosis (collagen deposition)	++++
Airway smooth muscle hypertrophy	+++
Mucus gland hyperplasia	+++

Lung tissue morphometry was scored by two investigators. A five-point semi-quantitative severity-based scoring system was used: 0: absence of alterations, +: alterations in 1–25% of the tissue examined, ++: alterations in 26–50% of the tissue examined, +++: alterations in 51–75% of the tissue examined, ++++: alterations in 76–100% of the tissue examined.

4. Discussion

In the present study, we developed a model of severe allergic lung inflammation with functional, histological, and immunological features that resembles severe human asthma. Histological analysis showed shedding and damage of airway epithelium, subepithelial fibrosis, bronchial smooth muscle hypertrophy, eosinophil and neutrophil infiltration in central and distal airways and lung parenchyma, and mucus gland hyperplasia, all of which significantly impaired baseline *in vivo* and *in vitro* lung mechanics. Furthermore, this protocol produces a high amount of Th2 cytokine (IL-4, IL-5, and IL-13), eosinophil and neutrophil infiltration in airways and lung parenchyma, and development of airway hyperreactivity.

Investigation of the pathophysiological mechanisms of severe asthma has been limited by the lack of a satisfactory animal experimental model. Models of severe allergic inflammation are based on the intensity of the inflammatory process as well as in airway hyperreactivity, rather than focused on the pulmonary pathologies representative of severe human asthma. In this line, Mukaiyama et al. (2004) analyzed the role of neurokinin NK3 receptor in a severe asthma guinea pig model characterized by increased number of eosinophils and airway hyperresponsiveness. However, these animals may yield exaggerated responses which are species-dependent and mediated by IgG rather than

Table 4
In vivo and *in vitro* mechanical parameters

	C	SA
<i>In vivo</i>		
Flow (ml s ⁻¹)	1.06 ± 0.02	1.05 ± 0.01
Volume (ml)	0.21 ± 0.01	0.20 ± 0.01
Est (cmH ₂ O ml ⁻¹)	17.90 ± 1.04	50.94 ± 5.25*
ΔP _{tot} (cmH ₂ O)	0.95 ± 0.02	1.88 ± 0.14*
ΔP _I (cmH ₂ O)	0.33 ± 0.02	0.47 ± 0.13*
ΔP ₂ (cmH ₂ O)	0.63 ± 0.02	1.41 ± 0.13*
<i>In vitro</i>		
E (10 ⁴ N/m ²)	1.10 ± 0.03	1.57 ± 0.05*
R (10 ² N s/m ²)	1.02 ± 0.04	1.21 ± 0.06*
η	0.06 ± 0.01	0.05 ± 0.01

Values are means (±S.E.M.) of eight animals in each group (10 determinations/animal). In severe allergic inflammation group (SA), mice were sensitized with ovalbumin and exposed to repeated challenges with intratracheal instillation of ovalbumin. Control group (C) received saline under the same protocol. All mechanical data were collected under baseline conditions. Est, ΔP_{tot}, ΔP_I, and ΔP₂=pulmonary static elastance, total, resistive and viscoelastic/inhomogeneous pressures, respectively. E, R, η= tissue elastance, resistance and hysteresivity, respectively.

* Significantly different from C group (*p* < 0.05).

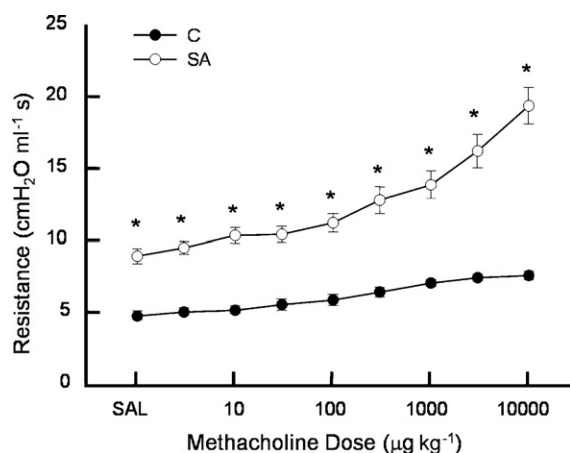


Fig. 3. Airway responsiveness in BALB/c mice after exposure to saline or OVA. Airway resistance was measured in response to increasing doses of methacholine. C = control group, SA = severe allergic inflammation group. Data are presented as means $\pm$ S.E.M. ($n = 8/\text{group}$). *Significantly different from C group ($p < 0.05$).

IgE, limiting their relevance to human allergic asthma (Karol, 1994; Kumar and Foster, 2002). Beavitt et al. (2005) observed that Lyn-deficient mice developed severe persistent asthma characterized by intense eosinophil-rich inflammation, hyper IgE, increased mucus production, overproduction of Th2 cytokines, mast cell and eosinophil hyperdegranulation, and deranged lung function. Even though these authors depicted many histological and functional data that resembled severe human asthma, airway remodelling characteristics were not described. In this context, Ochkur et al. (2007) using a double transgenic mouse model that expresses IL-5 systemically from mature T cells and eotaxin 2 locally from lung epithelial cells showed pulmonary pathologies representative of changes displayed by severe asthma patients. One important limitation of both studies (Beavitt et al., 2005; Ochkur et al., 2007) was the use of specific mice. While these models provided valuable information regarding contributions of potential mediators to specific aspects of disease pathology, prolonged allergen exposure triggered a distinct array of immunological responses that resulted in chronic inflammation and impact on airway integrity and function. In the current study, we used BALB/c mice, a strain commonly used in stud-

ies of allergic airway diseases. To develop the present model of severe allergic inflammation, pilot studies with different doses of ovalbumin during sensitization and challenges were performed in order to achieve some mechanical, histological and ultrastructural features that resemble severe human asthma. In the first study, we increased the dose of sensitization (20 μg) and associated it to an adjuvant (alum hydroxide), maintaining the challenge dose of 40 μg of ovalbumin. However, the mortality rate was high ($>80\%$) as well as the inflammatory process in airway and, mainly, lung parenchyma. In the second study, we augmented the dose of ovalbumin during challenge (60 μg), maintaining the sensitization dose at 10 μg of ovalbumin. In this case, the mortality rate reduced, but ultrastructural changes were not compatible with severe human asthma, instead resembling pneumonitis. Thus, we decided to reduce the challenge dose of ovalbumin sequentially from 60 to 40 μg analyzing the lung function and histology (light and electron microscopy). Therefore, the development of our model entailed intensive research and, consequently, we achieved a new level of fidelity of severe asthmatic lesions.

We observed that the contraction index and the fraction area of alveolar collapse were increased in SA group (Table 1, Fig. 2), suggesting that the inflammatory changes exist in the large airways as well as in the lung periphery, replicating characteristic features of severe asthma in human (Carroll et al., 1997; Balzar et al., 2005; de Magalhaes Simões et al., 2005) and animal models (Ochkur et al., 2007). In addition, we were able to observe that the numbers of eosinophils and neutrophils were increased both in lung parenchyma and BALF in SA group (Tables 1 and 2, Figs. 1 and 2). Electron microscopy showed eosinophil infiltration in peribronchiolar and perivascular regions, and in some animals neutrophils were in contact with the smooth muscle bundles (Fig. 2). Although neutrophils are involved in the innate immunity as the first group of cells to arrive in the injured local, they can participate in the Th2 inflammation response as in, for example, severe persistent asthma (Jatakanon et al., 1999; Kobayashi et al., 2005).

The method used for determination of pulmonary mechanics allows the identification of its elastic, resistive, and viscoelastic and/or inhomogeneous components (Bates et al., 1988). Baseline resistive pressure was higher in SA group compared to control (Table 4), probably due to airway constriction (Tables 1 and 5), bronchial oedema (Fig. 2), airway remodelling (Fig. 1) and subepithelial fibrosis (Fig. 2). Airway remodelling leads to decreased distensibility of the airway, exaggerated narrowing of the airway lumen when smooth muscle shortens, and irreversible airflow obstruction increasing the airway resistance (Ward et al., 2001). These findings are in agreement with previous clinical (Jeffery et al., 1992; Benayoun et al., 2003) and experimental (Ochkur et al., 2007) studies, showing that persistent airway inflammation and irreversible structural changes of the bronchial wall, defined as airway remodelling, play a prominent role in severe asthma. Increase in airway smooth muscle mass (Fig. 2 and Table 3) is of substantial importance for asthma pathophysiology because it could account for airway hyperresponsiveness (Lambert et al., 1993; An et al., 2007) and the permanent reduction of the airway calibre in severe

Table 5
Correlation matrix between physiologic and morphometric parameters

	Contraction index	Alveolar collapse (%)	Total cellularity (%)
Est, L ($\text{cmH}_2\text{O ml}^{-1}$)	0.64 (0.008)	0.66 (0.005)	0.71 (0.002)
ΔP_2 , L (cmH_2O)	0.74 (<0.001)	0.77 (<0.001)	0.76 (<0.001)
ΔP_1 , L (cmH_2O)	0.63 (0.009)	0.67 (0.004)	0.67 (0.004)
Collagen fibres ($\mu\text{m}^2/\mu\text{m}$)			
E (10^4 N/m^2)		0.75 (<0.001)	
R (10^2 N s/m^2)		0.53 (0.03)	

Est, ΔP_2 , ΔP_1 : pulmonary static elastance, viscoelastic/inhomogeneous, and resistive pressures, respectively; E : dynamic elastance. p values are shown in parentheses. The correlation was performed on data from control and severe allergic lung inflammation groups.

human asthma (Benayoun et al., 2003). Airway remodelling comprises a number of structural changes, including: epithelial detachment, subepithelial fibrosis, and smooth muscle hyperplasia and hypertrophy and these changes are probably related to the increase in IL-4, IL-5, and IL-13. Indeed, these cytokines plays a pivotal role in the pathogenesis of murine models of allergic asthma (Corry et al., 1996; Kuperman et al., 2002; Mojtavavi et al., 2002; Leigh et al., 2004) exerting various modulatory effects on bronchial hyperreactivity, inflammatory infiltration of eosinophils, and lung remodelling. In this line, many authors related the overexpression of these cytokines with the fibrotic response in the airway wall in patients with severe asthma (Zhu et al., 1999; Ingram et al., 2003). There was a significant increase in airway resistance for a given augmentation in methacholine dose in SA compared to the C group (Fig. 3). Baseline resistance in SA group was higher than control (Fig. 3), probably because of fixed airway obstruction (Fig. 1B and H). We also observed a relatively mild degree of maximal airway response to methacholine compared to that usually observed in acute animal models (Locke et al., 2007). These changes could be attributed to: (1) the rigidity of the airway avoiding constrictor response to agonist (Adler et al., 1998; McMillan and Lloyd, 2004), (2) mice are less sensitive to constrictor agonists than other species (Pretolani et al., 1994), (3) the absence of changes in elastic fibres content in airway and lung parenchyma as well as fragmentation of elastin (Mauad et al., 1999), and (4) the different response related to the route of administration (aerosol *versus* intravenous) (Nagase et al., 1994; Wagers et al., 2007). To clarify this issue, we analysed airway responsiveness to intravenous and inhaled methacholine and observed that the last intravenous injection of methacholine yielded a greater increase in airway resistance compared to inhaled methacholine (36%).

The current model also led to changes in lung static elastances and viscoelastic/inhomogeneous pressure (Table 4). There are controversies regarding the behaviour of elastic recoil in patients with severe asthma. Some patients present a loss of elastic recoil not caused by emphysematous changes, but probably determined by the loss of alveolar-airway attachments (Mauad et al., 2004). On the other hand, a reduction in lung compliance was also described (Liu et al., 1995). The augmentation in lung elastance and viscoelastic and/or inhomogeneous pressure suggests the presence of heterogeneities that can be due to many different factors, e.g., alveoli collapse, distortion of patent alveoli, and lung tissue infiltration with eosinophils and neutrophils (Table 5, Figs. 1 and 2). Indeed, autopsy (de Magalhaes Simões et al., 2005) and live patient studies with severe asthma (Balzar et al., 2002) reported increased distal lung inflammation. In addition, numerous investigators have argued in support of non-cellular mechanisms involved in lung mechanical changes. They described that impaired surfactant function, possibly resulting from interaction with proteins in inflammatory exudate, could lead to early and/or abnormal closure of small airways (Jarjour and Enhorning, 1999).

To better understand the role of lung parenchyma in the present model, tissue mechanics were analyzed. Physically, lung parenchyma can be simplified as a viscoelastic connective matrix connected to a contractile system that modulates its mechanical

properties. It is currently accepted that the connective tissue fibre network dominates parenchymal mechanics, together with a less significant role played by interstitial cells (Yuan et al., 1997; Lopez-Aguilar and Romero, 1998; Xisto et al., 2005). Tissue elastance and resistance were significantly increased in SA compared to control tissue. These *in vitro* mechanical changes were accompanied by deposition of collagen fibres with no modification in elastic fibre content. These data suggest that collagen fibres contributed to the differences in stiffness and in viscosity of lung tissue between SA and C groups. It is noteworthy, however, that not only the absolute amount of fibres is important in affecting mechanical behaviour, but also the organization and/or the interaction of these fibres. The absence of changes in tissue hysteresivity could be attributed to the non-disorganization in the extracellular matrix of lung parenchyma (Rocco et al., 2001). Thus, parenchymal mechanical dysfunction also plays an important role in the pathophysiology of the present model of severe allergic inflammation (Table 4).

In conclusion, this model of severe allergic lung inflammation replicates many of the features of severe human asthma, enables us to directly assess the role of airway and lung parenchyma inflammation and remodelling on respiratory mechanics, offering an opportunity to dissect the cellular and molecular mechanisms involved in these processes as well as facilitating studies of potential therapeutic agents. Indeed, no mouse model of asthma is able to mimic all of features of human disorders and caution is required to extrapolate to the human setting conclusions.

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