



## Different strains of mice present distinct lung tissue mechanics and extracellular matrix composition in a model of chronic allergic asthma

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### ABSTRACT

The impact of genetic factors on asthma is well recognized but poorly understood. We tested the hypothesis that different mouse strains present different lung tissue strip mechanics in a model of chronic allergic asthma and that these mechanical differences may be potentially related to changes of extracellular matrix composition and/or contractile elements in lung parenchyma. Oscillatory mechanics were analysed before and after acetylcholine (ACh) in C57BL/10, BALB/c, and A/J mice, subjected or not to ovalbumin sensitization and challenge. In controls, tissue elastance (*E*) and resistance (*R*), collagen and elastic fibres' content, and  $\alpha$ -actin were higher in A/J compared to BALB/c mice, which, in turn, were more elevated than in C57BL/10. A similar response pattern was observed in ovalbumin-challenged animals irrespective of mouse strain. *E* and *R* augmented more in ovalbumin-challenged A/J [*E*: 22%, *R*: 18%] than C57BL/10 mice [*E*: 9.4%, *R*: 11%] after ACh. In conclusion, lung parenchyma remodelled differently yielding distinct *in vitro* mechanics according to mouse strain.

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### 1. Introduction

Asthma is a chronic inflammatory disorder that involves the central airways as well as peripheral airways and the lung parenchyma (Tulic et al., 2001; Tulic and Hamid, 2003; Sutherland et al., 2004; Xisto et al., 2005). In an attempt to elucidate asthma pathogenesis and treatment (Ewart et al., 2000; Shinagawa and Kojima, 2003; Wagers et al., 2007), mice are increasingly used in the development of asthma models due to the availability of genetically characterized inbred strains, diversity of immunological tools (Kips et al., 2003; Zosky and Sly, 2007), and easy manipulation.

There is evidence that genetic strain variations in mice may exist with regard to *in vivo* respiratory mechanics and airway hyperresponsiveness (Tankersley et al., 1999; Ewart et al., 2000; Held and Uhlig, 2000; Reinhard et al., 2002; Ackerman et al., 2005; Wagers

et al., 2007). Recently, we showed that BALB/c and C57BL/10 mice present distinct tissue mechanical properties that were related to specific extra-cellular fibre composition and amount of contractile structures in their lung parenchyma, suggesting that genetic background determines basal lung tissue function and structural differences between these two strains (Faffe et al., 2006). So far, no study has described mouse strain variations in lung tissue mechanics and extracellular matrix components in a model of chronic allergic asthma.

The aim of this study was to test the hypothesis that different strains of mice present different *in vitro* lung mechanical profiles in a model of chronic allergic asthma and that these mechanical differences may be potentially related to changes of specific extracellular matrix composition and/or contractile elements in lung tissue.

### 2. Methods

#### 2.1. Animal preparation

Forty-two mice (25–30 g) were selected ( $n = 14$ /each) according to mouse strains (C57BL/10, BALB/c, and A/J). Each mouse strain was randomly divided into two main groups OVA and C groups. In OVA

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group, animals were immunized using an adjuvant-free protocol by the intraperitoneal injection of ovalbumin (OVA, 10  $\mu$ g) on each of 7 alternate days (Xisto et al., 2005). Forty days after the beginning of sensitization, 20  $\mu$ g of OVA in 20  $\mu$ l sterile saline were intratracheally instilled. This procedure was performed 3 times with a 3-day interval between them (OVA,  $n = 7$ /strain). The control group (C,  $n = 7$ /strain) received the same volume of saline.

Twenty-four hours after the last challenge, animals were sedated (diazepam 1 mg intraperitoneally), anesthetized (thiopental sodium: 20 mg/kg intraperitoneally) and heparine (1000 IU) was intravenously injected immediately before the section of abdominal aorta and vena cava. 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  $K_3PO_4$ , 25  $NaHCO_3$ , 2.5  $CaCl_2 \cdot H_2O$ , 0.6  $MgSO_4 \cdot H_2O$ , and 11.1 glucose] at pH 7.40 and 6 °C (Lopez-Aguilar and Romero, 1998; Rocco et al., 2001; Xisto et al., 2005).

## 2.2. Apparatus

Parenchyma strips (2 mm  $\times$  2 mm  $\times$  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%  $O_2$ –5%  $CO_2$ . Lung strips were weighed ( $W$ ), and their unloaded resting lengths ( $L_0$ ) were determined with a calliper. Lung strip volume was measured by simple densitometry, as:  $vol = \Delta F / \delta$ , where  $\Delta F$  is the total change in force before and after strip immersion in K–H solution and  $\delta$  is the mass density of K–H solution (Lopez-Aguilar and Romero, 1998; Romero et al., 2001). One end of the strip was attached to a force transducer (LETICA TRI-110, Scientific Instruments, Barcelona, Spain), whereas the other one was fastened to a lever arm actuated by means of a modified woofer driven by the signal generated by a computer and analogue-to-digital converted (AT-MIO-16-E-10, National Instruments, Austin, TX, USA). A sidearm of this rod was linked to a second force transducer (LETICA TRI-110, Scientific Instruments, Barcelona, Spain) by means of a silver spring of known Young's modulus, thus allowing the measurement of displacement. Neither amplitude dependence (<0.1% change in stiffness) nor phase changes with frequency were detected in the range from 0.01 to 14 Hz.

## 2.3. Preconditioning

Cross-sectional, unstressed area ( $A_0$ ) of the strip was determined from volume and unstressed length, according to  $A_0 = vol / L_0$ . Basal force ( $F_B$ ) for a stress of 0.1 N/cm<sup>2</sup> was calculated as  $F_B (N) = 0.1 (N/cm^2) \cdot A_0 (cm^2)$  and adjusted by vertical displacement of the force transducer as described before (Romero et al., 2001). The displacement signal was then set to zero. Once basal force and displacement signals were adjusted, the length between bindings ( $L_B$ ) was measured by means of a precision calliper. Instantaneous length during oscillation around  $L_B$  was determined by adding the value of  $L_B$  to the measured value of displacement at any time. Instantaneous average cross-section area ( $A_i$ ) was determined as  $A_i = Vs / L_i$  (cm<sup>2</sup>). Instantaneous stress ( $\sigma_i$ ) was calculated by dividing force ( $g$ ) by  $A_i$  (cm<sup>2</sup>). Strain was calculated as  $\Delta \epsilon = (L - L_B) / L_B$ . Each parenchyma strip was preconditioned for 30 min by sinusoidal oscillation of the tissue (frequency = 0.5 Hz; amplitude large enough to reach a final stress of 0.2 N/cm<sup>2</sup>). Thereafter, the amplitude was adjusted to 5%  $L_0$  and the oscillation maintained for another 30 min, or until a stable length-force loop was reached.

## 2.4. Experimental protocol

After preconditioning, the strips were oscillated at a frequency ( $f$ ) = 1 Hz and tissue elastance ( $E$ ) and resistance ( $R$ ) were calculated (Fredberg and Stamenovic, 1989). Three baseline recordings (20 s

duration) were done. Acetylcholine (ACh) solution, in K–H warmed at 37 °C, was then added to the bath to reach successive concentrations of  $10^{-5}$ ,  $10^{-3}$ , and  $10^{-1}$  M. Signals were recorded at 30 s, 1, 2, and 3 min after each dose (20 s duration each). Both force and displacement signals were preamplified, filtered at 30 Hz (902LPF Frequency Devices, Haverhill, MA, USA), analogue-to-digital converted (AT-MIO-16E-10, National Instruments Co., Marlboro, MA, USA) and sampled at a frequency of 150 Hz (Software LabVIEW 5.1, National Instruments Co., Austin, TX, USA).

## 2.5. Morphometric analysis

Lung tissue strips were fixed with 4% paraformaldehyde. After fixation, the tissue was embedded in paraffin. Blocks were cut 4  $\mu$ m-thick by a microtome. For morphometric measurements, slices were stained with haematoxylin-eosin and analysed with an integrated eyepiece with a coherent system made of a 100-point and 50-lines grid of known length coupled to a light microscope (Axioplan, Zeiss, Oberkochen, Germany). Sections were examined at 400 $\times$  magnification, and the fractional areas of alveolar wall (AW), blood-vessel wall (BVW), and bronchial wall (BW) were determined by the point-counting technique (Weibel, 1990). All points falling on these components were counted and divided by the total number of points. This analysis was performed in 10 random, non-overlapping fields in each strip. BVW and BW were counted when a point fell on the endothelial and epithelial layers, the smooth muscle, or associated connective tissue. Points falling on alveolar air spaces, blood-vessel lumen, and bronchial lumen were excluded.

Tissue slices also underwent specific staining methods to compute the amount of collagen and elastic fibres in the alveolar septa. For collagen, the tissue was stained in a solution of Sirius red dissolved in aqueous saturated picric acid and observed under polarized light microscopy (Montes, 1996). For elastic fibres, the Weigert's resorcin fuchsin method modified with oxidation was used (Fullmer et al., 1974). In each strip, 20 different microscopic fields were randomly selected to quantify collagen and elastic fibres. Quantification (200 $\times$  magnification) was carried out with the aid of a digital analysis system, using specific software (Bioscan-Optimas 5:1, Bioscan, Edmond, WA, USA). The images were generated by a microscope (Axioplan, Zeiss, Oberkochen, Germany) connected to a camera (Trinitron CCD, Sony, Tokyo, Japan) and fed into a computer through a frame grabber (Oculus TCX, Coreco, St. Laurent, Que., Canada) for off-line processing. The thresholds for collagen and elastic fibres were established after enhancement of the contrast up to the point where the fibres were easily identified as either black (elastic) or birefringent (collagen) bands. Bronchi and blood vessels were carefully avoided during the measurements. The areas occupied by the elastic and collagen fibres, measured in each alveolar septum, were divided by the length of the corresponding septum. The results were expressed as the number of elastic and collagen fibres per unit of septum length.

## 2.6. Smooth-muscle-specific actin evaluation

Immunohistochemical staining was performed in slides 5  $\mu$ m-thick of lung tissue strips fixed with 4% paraformaldehyde, using monoclonal antibody to  $\alpha$ -smooth muscle actin (Dako, Carpinteria, CA, USA) at a 1:500 dilution. Sections were then rinsed with Tris-buffered saline and incubated sequentially with biotinylated rabbit antimouse IgG (Dako Corp., Cambridge, UK) at a dilution of 1:400, followed by streptavidin combined *in vitro* with biotinylated horseradish peroxidase at a dilution of 1:1000 (Dako, Cambridge, UK). The reaction product was developed using diaminobenzidine tetrahydrochloride. Sections were counterstained with haematoxylin for 1 min, dehydrated through graded alcohols, and mounted in resinous mountant. Known positive con-

trols were included with each run, and negative controls had the primary antibody omitted (Dolhnikoff et al., 1998). The analysis was performed on the slides stained for actin applying the point-counting technique (Weibel, 1990). Using a 121-point grid, we calculated the volume proportion of smooth-muscle-specific actin in terminal bronchioles and alveolar ducts as the relation between the number of points falling on actin-stained and non-stained tissue. Measurements were done at 400 $\times$  in each slide.

### 2.7. Statistical analysis

SigmaStat 3.0 statistical software package (Jandel Corporation, CA, USA) was used. ANOVA followed by Tukey test was applied to test the differences between C and OVA groups in different mouse strains. To analyse the effect of Ach in lung mechanics, paired t test was used. Correlation between mechanical and histological data was determined by Spearman correlation test.  $p$ -value <0.05 was considered significant.

## 3. Results

Fig. 1 shows values of the oscillatory mechanics measured before (basal) and after acetylcholine (ACh) challenge in control (C) and ovalbumin (OVA) lung tissue strips obtained from C57/BL10, BALB/c, and A/J mice. Tissue elastance ( $E$ ) and resistance ( $R$ ) were higher in A/J than in BALB/c and C57BL/10 mice ( $p < 0.05$ ) independent of the

study group.  $E$  and  $R$  were measured at 30 s, 1, 2, and 3 min after ACh treatment, however, the time point presenting the highest value of  $R$  was used only for subsequent analysis of all mechanical parameters.  $E$  and  $R$  increased in all groups after  $10^{-1}$  M ACh ( $p < 0.05$ ) with percentage increase in  $E$  and  $R$  higher in A/J [ $E$ : 21%,  $R$ : 18%] than BALB/c [ $E$ : 15%,  $R$ : 16%] and C57BL/10 [ $E$ : 9%,  $R$ : 10%] mice in OVA groups. All mechanical parameters were higher in OVA than in C group irrespective of the mouse strain, and A/J asthmatic mice presented higher  $E$  and  $R$  compared to the other strains (Fig. 1).

Table 1 shows the results of morphometrical analysis. All groups showed similar contents of bronchial, blood vessel, and alveolar walls. However, the amount of collagen and elastic fibres (Table 2) and the volume proportion of  $\alpha$ -actin in the lung periphery (Table 3) were higher in A/J than BALB/c and C57BL/10 mice. Fig. 2 shows the increased content of  $\alpha$ -actin, collagen and elastic fibres in the A/J (Fig. 2C, F, and I, respectively) compared to C57/BL10 and BALB/c mice in OVA groups.

Fig. 3 depicts the correlations between tissue resistance and elastance data at 1 Hz before acetylcholine challenge and morphological data.  $E$  and  $R$  were significantly correlated with the amount of collagen and elastic fibres and with the volume proportion of  $\alpha$ -actin (Fig. 3).

## 4. Discussion

In the present murine model of chronic allergic asthma A/J mice presented higher amount of collagen and elastic fibres and  $\alpha$ -actin in distal airways and lung parenchyma than BALB/c and C57BL/10 mice associated with an increase in tissue elastance and resistance.

Different mouse strains have been studied to investigate the genetic mechanisms of asthma-related phenotypes associated with allergen exposure. Murine models have many advantages such as genetic homogeneity within inbred populations, environmental homogeneity, and the possibility to apply a uniform allergic stimulus (Ewart et al., 2000). We have previously compared the lung parenchyma mechanics between BALB/c and C57BL/10 mice in normal conditions, and observed that the former was more responsive than the latter (Faffe et al., 2006). In our current study, we analysed the response, both in baseline conditions and after allergen-challenge, in A/J, BALB/c and C57BL/10 mice. These strains were selected due to their frequent use in lung physiologic and immunologic investigations (Held and Uhlig, 2000). In this line, BALB/c mice are often used in laboratory animal research for immunologic examinations, while C57BL/10 mice serve as a background strain of many genetically engineered mice, and A/J mice are chosen due to their increased reactivity to mediators, such as acetylcholine (Shinagawa and Kojima, 2003; Ackerman et al., 2005; Kim et al., 2007).

Previous data compared lung function among different mouse strains by approaching only central airway alterations or the whole respiratory system characteristics (Tankersley et al., 1999; Schulz et al., 2002; Reinhard et al., 2002; Shinagawa and Kojima, 2003; Kim et al., 2007; Wagers et al., 2007). The absence of reports regarding the rheological properties of living pulmonary tissue in different mouse strains led to the decision to analyse *in vitro* lung mechanics and histology, both in control and in an experimental model of chronic asthma in A/J, BALB/c and C57BL/10 mice.

Lung parenchyma is a viscoplastoelastic material and different anatomic mechanisms have been proposed to account for this behaviour, such as: extracellular matrix, the surface film, and the contractile apparatus. The extracellular matrix consists of collagen and elastic fibres, proteoglycans and glycosaminoglycans (Fredberg et al., 1993; Mijailovich et al., 1993; Cavalcante et al., 2005). Tissue elastance and resistance of lung parenchyma in OVA group were significantly increased compared to controls, suggesting that

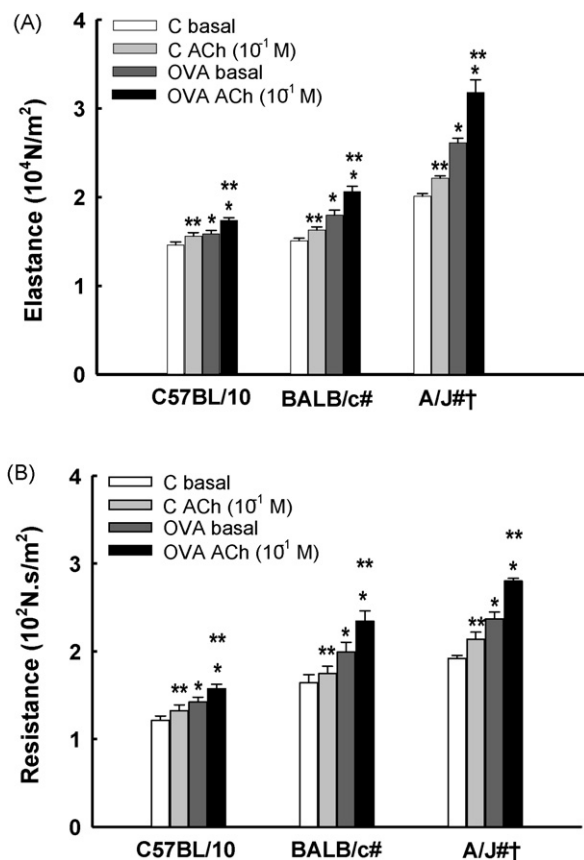


**Fig. 1.** Oscillatory mechanics [tissue elastance (A) and resistance (B)] at a frequency of 1 Hz measured before (basal) and after acetylcholine (ACh,  $10^{-1}$  M) challenge in control (C) and ovalbumin (OVA) lung tissue strips obtained from C57/BL10, BALB/c, and A/J mice. Values are means  $\pm$  SEM of seven animals in each group. \*Significantly different from C group ( $p < 0.05$ ). \*\*Significantly different from basal ( $p < 0.05$ ). #E and R were significantly higher in A/J and BALB/c compared to C57BL/10 independent of study group ( $p < 0.05$ ). †E and R were significantly higher in A/J compared to BALB/c independent of study group ( $p < 0.05$ ).

**Table 1**  
Morphometrical parameters.

|         | C57BL/10     |              | BALB/c       |              | A/J          |              |
|---------|--------------|--------------|--------------|--------------|--------------|--------------|
|         | C            | OVA          | C            | OVA          | C            | OVA          |
| AW (%)  | 95.76 ± 0.68 | 95.26 ± 0.85 | 95.69 ± 1.26 | 94.87 ± 2.56 | 95.51 ± 0.85 | 95.82 ± 1.77 |
| BVW (%) | 1.95 ± 0.31  | 1.86 ± 0.37  | 1.73 ± 0.91  | 2.16 ± 0.96  | 1.62 ± 0.18  | 1.67 ± 0.34  |
| BW (%)  | 2.29 ± 0.62  | 2.88 ± 0.93  | 2.57 ± 1.36  | 2.96 ± 2.11  | 1.87 ± 0.75  | 2.51 ± 1.72  |

Values are means ± SEM of 7 animals in each group. AW, BVW and BW, volume proportions of alveolar, blood vessel, and bronchial wall, respectively.

**Table 2**  
Collagen and elastic fibre content in lung parenchyma strips.

|                                                | C57BL/10      |                | BALB/c         |                   | A/J               |                    |
|------------------------------------------------|---------------|----------------|----------------|-------------------|-------------------|--------------------|
|                                                | C             | OVA            | C              | OVA               | C                 | OVA                |
| Collagen fibre ( $\mu\text{m}^2/\mu\text{m}$ ) | 0.013 ± 0.001 | 0.019 ± 0.001* | 0.028 ± 0.003# | 0.051 ± 0.004*.*# | 0.044 ± 0.005#    | 0.065 ± 0.003*.*.# |
| Elastic fibre ( $\mu\text{m}^2/\mu\text{m}$ )  | 0.272 ± 0.007 | 0.278 ± 0.005  | 0.333 ± 0.007# | 0.337 ± 0.008#    | 0.399 ± 0.010*.*# | 0.517 ± 0.043*.*.# |

Values are means ± SEM of 7 animals in each group.

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

\*\* Significantly different from BALB/c group ( $p < 0.05$ ).

# Significantly different from C57BL/10 ( $p < 0.05$ ).

**Table 3**  
Volume proportion of smooth-muscle-specific actin in alveolar ducts and terminal bronchioles among different mouse strains.

|                                         | C57BL/10    |               | BALB/c        |                  | A/J           |                  |
|-----------------------------------------|-------------|---------------|---------------|------------------|---------------|------------------|
|                                         | C           | OVA           | C             | OVA              | C             | OVA              |
| Alveolar Ducts $\alpha$ -actin (%)      | 4.13 ± 0.53 | 5.94 ± 0.40*  | 7.91 ± 0.52#  | 13.61 ± 0.75*.*# | 6.61 ± 0.33#  | 15.73 ± 1.36*.*# |
| Terminal Bronchiole $\alpha$ -actin (%) | 9.49 ± 0.74 | 13.11 ± 1.06* | 16.36 ± 1.58# | 26.26 ± 2.54*.*# | 18.71 ± 1.77# | 28.69 ± 1.78*.*# |

Values are means ± SEM of 7 animals in each group.

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

# Significantly different from C57BL/10 ( $p < 0.05$ ).

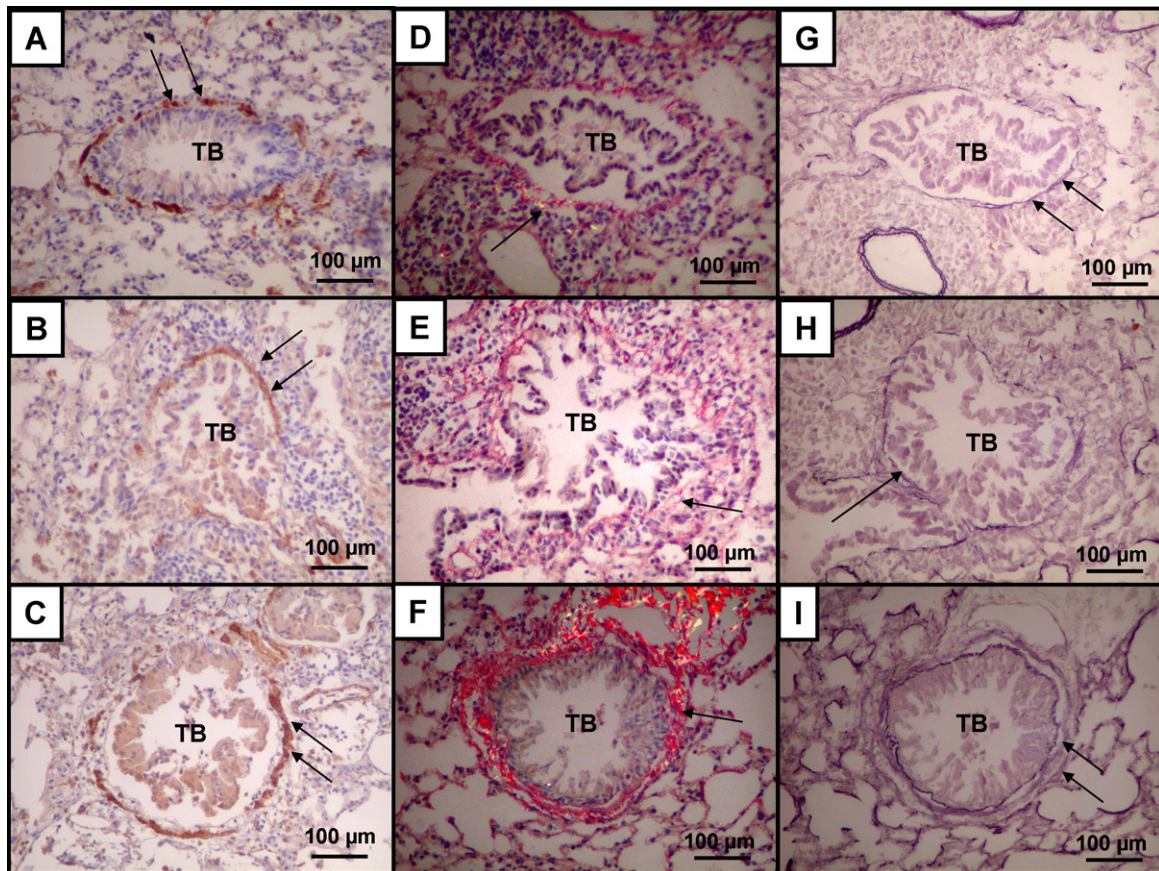


**Fig. 2.** Representative photomicrographs of parenchymal strips in C57BL/10 (A), BALB/c (B) and A/J (C) mice in OVA groups. A–C panels: Immunostaining for  $\alpha$ -smooth muscle actin in OVA strip. Note positive staining for actin in terminal bronchiole (TB) (arrows). D–F panels: OVA strip stained with Sirius Red with polarization for collagen. Collagen fibres are seen in bright orange (arrows). Note large area of fibrosis in A/J mice (F). G–I panels stained with Weigert's Resorcin-fuchsin with oxidation. Elastic fibres are seen in black (arrows). Note increased amount of elastic fibres (arrow) within fibrotic area. Scale bars = 100  $\mu\text{m}$ .

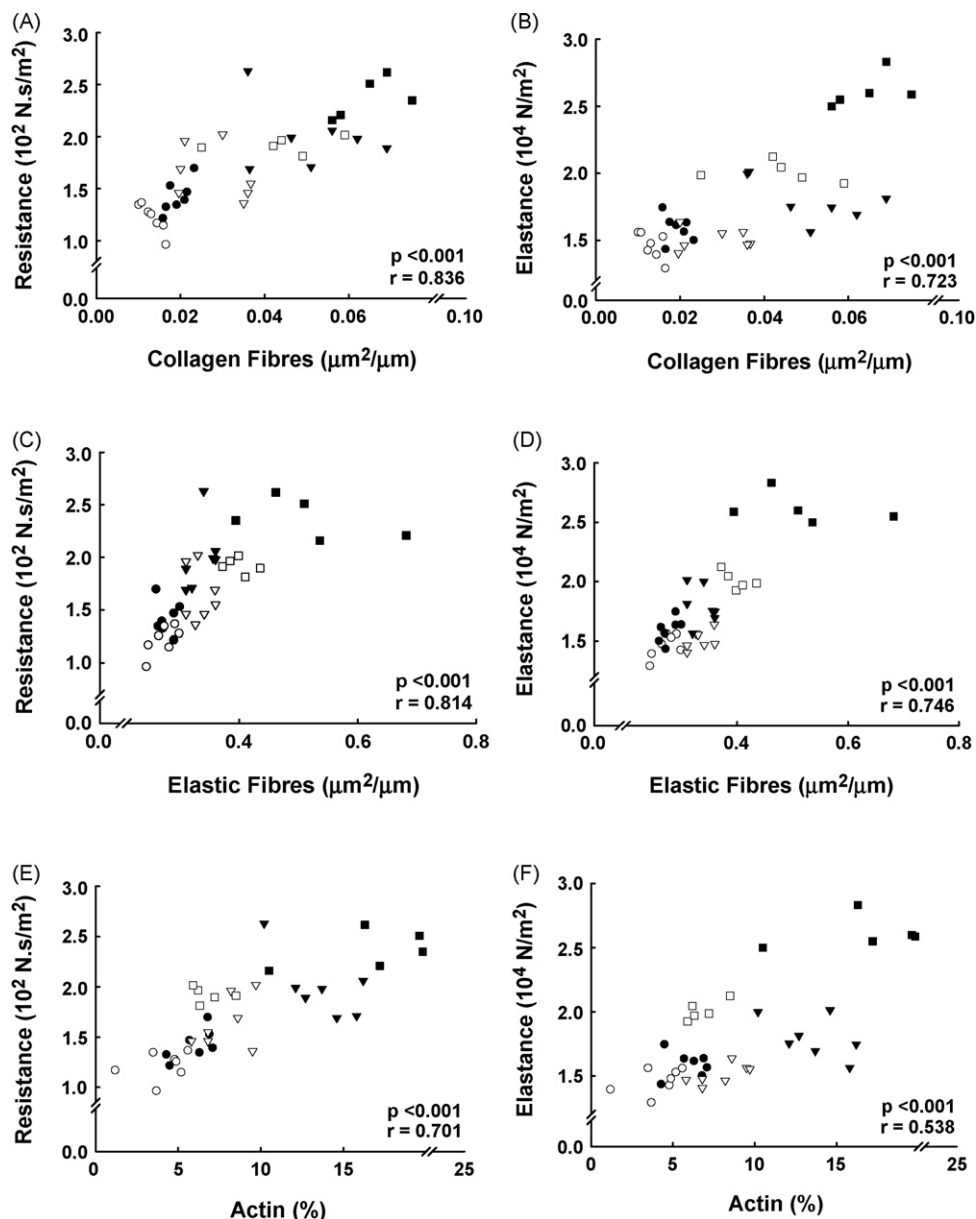


**Fig. 3.** Correlations between resistance (A, C and E) and tissue elastance (B, D and F) data at 1 Hz before acetylcholine challenge and morphological data obtained by Spearman's correlation test among C57BL/10 (circles), BALB/c (triangles) and A/J (squares) mice, between C (white symbols) and OVA (black symbols) groups.

parenchymal mechanical dysfunction plays an important role in the pathophysiology of asthma according to this model. These changes in tissue mechanics were accompanied by deposition of collagen fibres (Table 2) and increase in volume proportion of smooth-muscle-specific actin (Table 3) in C57BL/10 and BALB/c mice. In A/J mice these mechanical modifications were also associated with a high amount of elastic fibre. Additionally, we demonstrated that A/J mice have a greater amount of collagen and elastic fibres in comparison to BALB/c and C57BL/10 mice in control and OVA groups, suggesting that collagen and elastic fibres contributed to the differences in stiffness and in viscosity of lung tissue among the three strains (Mijailovich et al., 1993). Small airways and small blood vessels may also contribute to the observed changes in peripheral mechanics (Kapanci et al., 1974). However, in our current study no significant modifications in the amount of small blood vessels and small airways in lung parenchyma were observed (Table 1).

In the present study, ACh was used to induce smooth muscle contraction. There is evidence that airway epithelium presents acetylcholinesterase activity, which could attenuate the airway

smooth muscle contractile response (Ohruai et al., 1991; Koga et al., 1992). We analysed lung tissue strip, which presents low amount of airway epithelial cells. Furthermore, in allergic airway inflammation the epithelial shedding may down-regulate the cholinergic system (Lips et al., 2007), maintaining the contractile responses. The addition of ACh to the organ bath resulted in higher values of  $E$  and  $R$  in different mouse strains (Dolhnikoff et al., 1998; Fust et al., 2004; Salerno et al., 2004; Faffe et al., 2006). In our *in vitro* preparation, we demonstrated that A/J mice are more responsive to ACh than BALB/c and C57BL/10 mice both in control and sensitized mice, in accordance with the previous *in vivo* studies (Duguet et al., 2000; Shinagawa and Kojima, 2003; Wagers et al., 2007) (Fig. 1). These changes could be attributed to the amount of the contractile cells that include smooth muscle cells in the alveolar duct and vessel walls and myofibroblasts and fibroblasts in the interstitium. However, a higher constrictor activity of ACh may also be related to changes in receptor protein level or intracellular signaling due to chronic inflammation. Stimulation of contractile cells with ACh induces local internal stresses in the fibre network which can lead

to changes in the viscoelastic properties of the lung tissue. Furthermore, a higher constrictor activity of ACh may be related not only to the amount of  $\alpha$ -actin as previously reported (Dolhnikoff et al., 1998; Romero et al., 2001) but also to increased elastic and collagen fibre content in lung parenchyma. Together, these changes may lead to a rearrangement of extracellular matrix components and different airway-parenchyma interdependence (Ludwig, 1997; Romero et al., 2001; Cavalcante et al., 2005).

In conclusion, in the present murine model of chronic allergic asthma, lung parenchyma remodelled differently and presented distinct tissue mechanics according to mouse strain. Our results are important since they can orientate future studies in the choice of mouse strain according to the research objective.

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