



Allergen-induced asthma in C57Bl/6 mice: Hyper-responsiveness, inflammation and remodelling

Valentina Pinelli, Cinzia L. Marchica, Mara S. Ludwig*

Meakins Christie Laboratories, McGill University Hospital Centre, 3626 St. Urbain St., Montreal, PQ, Canada H2X 2P2

ARTICLE INFO

Article history:
Accepted 10 August 2009

Keywords:

Impedance
Mucus
Collagen
Biglycan
Decorin
Airway smooth muscle

ABSTRACT

The relationship among airway responsiveness, inflammation and remodelling in asthma is incompletely understood. To investigate potential mechanistic associations, allergen-induced asthma was studied in C57Bl/6 mice. Mice were sensitized and challenged with ovalbumin (OVA) using sub-acute (SA) or chronic (C) protocols. Responsiveness was assessed by measuring respiratory impedance which was partitioned into airway resistance (Raw) and distal lung components (Gti, Hti) during methacholine-induced constriction. Inflammation, airway mucus, airway smooth muscle, collagen, biglycan and decorin were quantified. The airways were sub-divided into central or peripheral. In SA and COVA, Raw, Gti and Hti responsiveness were significantly increased; the peripheral response was significantly greater in SA vs C OVA. Airway inflammation and mucus were increased in both groups, but more significantly in peripheral airways in SA OVA. In the SA OVA model, inflammation and mucus appear to drive the mechanical response, especially in the lung periphery; airway remodelling seems to contribute to hyper-responsiveness to an equivalent degree, after both challenge protocols.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

The mouse model of allergen-induced asthma is commonly used to investigate questions related to the link among airway hyper-responsiveness, inflammation and remodelling. Different approaches involve delivery of allergen in acute or more chronic challenge protocols. Recent data published by Southam et al. (2007) and Locke et al. (2007) showed that more acute challenge leads to airway inflammation, while more chronic challenge results in airway wall remodelling. The precise link between these changes and the degree of induced airway responsiveness, however, remains controversial.

Asthma is a disease characterized by heterogeneous airway constriction, which is particularly prominent in the lung periphery. The contribution of the distal lung to asthma pathology has been an area of recent interest (Kraft, 1999). It is possible that some of the difficulty in correlating changes in responsiveness with

changes in inflammation and remodelling arises because published studies do not separate out changes in more central and distal airways. For example, a recent report by Hirota et al. (2006), showed that changes in the extracellular matrix components, collagen and fibronectin, were not seen at the distal lung level.

The studies cited above investigated allergen challenge in BALB/c mice, a strain that has been reported to be intermediate in its sensitivity to allergen-induced pathology (Shinagawa and Kojima, 2003; Whitehead et al., 2003). Whether similar findings would be evident in other mouse strains is not evident. C57Bl/6 mice are a strain commonly used in genetic modification. Some data are reported in the literature using allergen challenge in this strain; however, the results are varied. Whereas some studies show minimal changes in indices of responsiveness, inflammation or remodelling (Shinagawa and Kojima, 2003; Whitehead et al., 2003), a recent study by Lee et al. (2004) demonstrated significant eosinophilia and airway hyper-responsiveness in this mouse strain.

In order to investigate potential links among responsiveness, inflammation and remodelling, we employed sub-acute and chronic allergen challenge protocols. We measured lung impedance and methacholine responsiveness, partitioning changes into central airway resistance and a more distal or peripheral lung response (tissue damping and elastance). We characterized changes in lung inflammation, mucus secretion, airway smooth muscle and extracellular matrix remodelling, and separated out these indices into central and distal airway components. Finally, we carried out this experiment in the C57Bl/6 murine strain.

Abbreviations: ASM, airway smooth muscle; BAL, bronchoalveolar lavage; Gti, damping; Hti, elastance; I, inertance; MCh, methacholine; OVA, ovalbumin; P, pressure; P_{bm}, basement membrane perimeter; PEEP, positive end expiratory pressure; Ptp, transpulmonary pressure; Raw, airway resistance; V, volume; Z_{RS}, respiratory system impedance.

* Corresponding author. Tel.: +1 514 398 3864; fax: +1 514 398 7483.

E-mail addresses: maraludwig@mcgill.ca, nicole.ryan@mail.mcgill.ca (M.S. Ludwig).

2. Materials and methods

2.1. Animal preparation

Male and female C57Bl/6 mice were purchased from Charles River Laboratories (Saint-Constant, Quebec, Canada). Animals between the ages of 6–8 weeks were studied. All animals received humane care in compliance with the Guide to the Care and Use of Experimental Animals formulated by the Canadian Council of Animal Care; procedures were reviewed and approved by an institutional animal ethics committee of McGill University.

2.2. Ovalbumin sensitization and challenge

Sensitization and challenge protocols for the different groups in this study are summarised in Fig. 1. Mice were sensitized by intraperitoneal (ip) injection of 10 µg ovalbumin (OVA) (Sigma Chemical Co., St Louis, MO) and 1 mg aluminium hydroxide (Fisher Scientific Ottawa, ON). Mice were sensitized on two occasions, seven days apart. One week after the second sensitization, mice were challenged intra-nasally, under light halothane anesthesia, by intra-nasal instillation of 10 µg OVA. Three groups of mice were studied. In the sub-acute (SA) group, OVA was delivered 3 times/wk for 3 weeks, and mice studied at day 35. A second group was studied at day 46, permitting recovery (R) from the sub-acute challenge. A third group of mice received 7 additional challenges between days 41 and 54, and were studied at day 57 (C). Control mice for all three groups were sensitized and challenged with saline (SAL).

2.3. Measurement of lung mechanics during methacholine challenge

Mice were injected ip with xylazine (10 mg/kg) and pentobarbital sodium (30 mg/kg). Mice were tracheostomised and an 18 gauge cannula was inserted into the trachea. Mice were connected to a computer controlled small animal ventilator (Flexivent, Scireq Inc., Montreal, Canada) and mechanically ventilated at 150 breaths/min, tidal volume of 10 ml/kg, PEEP of 3 cm H₂O (Alcorn et al., 2007). Animals were then paralyzed with pancuronium bromide (1.2 mg/kg ip).

The forced oscillation technique was used to measure respiratory mechanics. Regular ventilation was interrupted and a computer generated volume signal that consisted of waveforms of mutually primed frequencies was delivered to the airway opening. Piston displacement and cylinder pressure were measured.

Impedance values were obtained before and after the delivery of increasing concentrations of methacholine aerosols. Prior to the start of the methacholine concentration response curve, two total lung capacity breaths were delivered. Methacholine (Sigma) dissolved in saline was given via an ultrasonic nebuliser (Hudson RCI, Teleflex Medical) in increasing concentrations (6.25, 12.5, 25 and 50 mg/ml). After delivery of each aerosol, forced oscillations were delivered every 15 s, over a 5-min duration. In between impedance measurements, regular ventilation was resumed. The peak response for each variable was determined.

2.4. Calculation of mechanical parameters

Respiratory system impedance (Z_{RS}) was determined using the equation:

$$Z_{RS}(\omega) = \frac{P(\omega)}{[dV(\omega)/dt]}$$

where P is pressure and V is piston displacement volume (ml). Both are functions of angular frequency (ω).

Multiple linear regression was used to fit respiratory system impedance to the constant phase model of the lung (Hantos et al., 1992) using the equation:

$$Z_{RS} = R_{aw} + j\omega I + Gti + \frac{jHti}{\omega^\alpha}$$

where R_{aw} is airway resistance, I is airway inertance, Gti is tissue damping, Hti is tissue elastance, j is the imaginary unit and $\alpha = (2/\pi)\tan^{-1}(H/G)$. Values of R_{aw} at the highest concentration of MCh are not reported, as at this high level of induced constriction, significant heterogeneities in airway constriction were introduced and many negative values for R_{aw} were recorded. Only model fits that resulted in a coefficient of determination >0.80 were used.

2.5. Bronchoalveolar lavage

After lung mechanics measurements were completed, bronchoalveolar lavage (BAL) was performed by two intra-tracheal instillation of 1 ml ice-cold saline. Total cells in the BAL were counted using a hemocytometer. Differential counts of eosinophils, neutrophils, lymphocytes, and monocytes were determined on cytospin slides fixed and stained with Diff-Quick (Fisher Scientific, Kalamazoo, MI). Three hundred cells were counted. Results are expressed as total cells number $\times 10^4$.

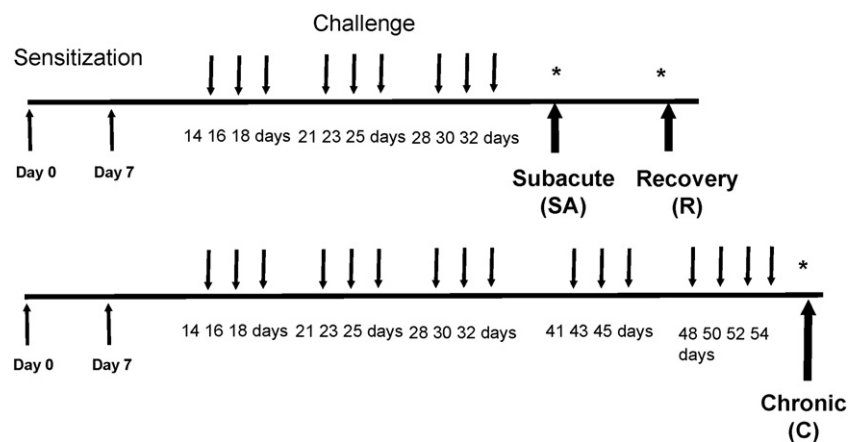


Fig. 1. Schematic of experimental protocol. Three groups of ovalbumin challenged mice were studied: sub-acute challenge (SA) (35 days); recovery (R) (46 days) and chronic challenge (C) (57 days). Control mice were sensitized and challenged with saline at the equivalent timepoints.

2.6. Tissue preparation and fixation

Following these measurements, the chest wall was opened and lungs were excised. The lungs were inflated with 10% phosphate-buffered formalin at 25 cm H₂O Ptp for 25 min. The lungs were then placed in 10% formalin overnight. The left lung was placed in a tissue processor. After processing, lungs were embedded in paraffin blocks and sagittal tissue sections cut (5 μ m).

2.7. Histochemistry

Hematoxylin and eosin (H&E): In order to evaluate tissue inflammation, hematoxylin and eosin staining was performed on paraffin embedded sections. Tissue sections were rehydrated and then immersed in hematoxylin (Gill II hematoxylin, Sigma–Aldrich) for 3 min. Subsequently, slides were rinsed with water, followed by saturated lithium carbonate, and then water again. Next, slides were immersed in 1% eosin (Sigma–Aldrich) for 1 min, after which the tissue sections were dehydrated, and mounted in resinous media (Cytoseal™ 60 mounting medium, Low viscosity, Co. Richard–Allen Scientific, Kalamazoo, MI) and cover slips applied.

Periodic-Acid Schiff (PAS): Mucus secretion was visualized using Periodic-acid Schiff staining. Paraffin embedded lung tissue sections were de-waxed and hydrated. Following rehydration, tissue sections were treated with Periodic-acid Solution (Aqueous solution, 1 g/l, Sigma–Aldrich) for 10 min. After washing repeatedly in water, slides were covered with Schiff's reagent solution (pararosaniline HCl, 1% (w/v) Sodium bisulfite, 4% (w/v) in HCl, 0.25 mol/l, Sigma–Aldrich) for 15 min. Slides were then washed in water and immersed in Harris Hematoxylin, followed by a differentiation in acid alcohol (HCL concentration 4 ml; 95% EthOH–396 ml). Sections were washed in water, followed by lithium carbonate, followed by water once more. The tissue sections were then dehydrated through different concentrations of alcohol, and sections were mounted in Cytoseal and cover slips applied.

Picro-sirius red: This stain was used to visualize collagen within lung tissue sections. Tissue sections from both control and OA challenged animals were de-waxed and hydrated. The tissue sections were subsequently immersed in picro-sirius red solution (Sirius Red F3B 0.5 g; saturated aqueous solution of picric acid–500 ml; solid picric acid; Sigma–Aldrich) for 1 h. The tissue sections were then washed in two changes of acidified water (0.5% glacial acetic acid), followed by dehydration through alcohol. Sections were then cleared in Xylene, mounted in Cytoseal and cover slips applied.

2.8. Immunohistochemistry

Alpha-smooth muscle actin: Antigen retrieval was initially performed on tissue sections using sodium citrate buffer 0.01 M (pH 6.0). Sections were then incubated overnight at 4 °C in a humid chamber with the primary antibody α -smooth muscle actin (1:250 dilution, rabbit anti-mouse α -sma, Abcam Laboratories, Cambridge, MA). The secondary antibody applied was biotinylated goat anti-rabbit (1:100 dilution, Vector Laboratories), and immunoperoxidase was used for detection. Slides were counterstained using hematoxylin (Gill II Hematoxylin, Sigma–Aldrich). All immunostaining was developed using DAB. Positive stain for α -SMA was visualized to be brown under a light microscope (Olympus BX51, ON, Canada).

Decorin and Biglycan: Tissue sections were incubated overnight with the primary antibody for decorin (rabbit anti-decorin 1:800 dilution), or biglycan (rabbit anti-biglycan 1:500 dilution, both provided by Dr. Larry Fisher, NIH). The biotinylated secondary antibody used was goat anti-rabbit (1:100 dilution, Vector Laboratories). Immunoperoxidase was used for detection. Slides were then counterstained using hematoxylin (Gill II

Hematoxylin, Sigma–Aldrich). All immunostaining was developed with DAB.

2.9. Morphometry

Eight mice were examined per group and five airways per mouse were chosen randomly (excepting PASs, where 3 airways/mouse were examined). Airway selection criteria included an intact epithelial lining and a minimum to maximum internal diameter ($D2/D1$) ≥ 0.5 . Slides were examined with a light microscope and images were captured with either an Olympus Qcolor5 Camera (Olympus, Center Valley, PA) or a Cool-Snap PROcf color camera (Media Cybernetics, Baltimore, MD) and Image pro PLUS software (Version 5.2, Media cybernetics L.P., Silver Spring, MD, U.S.A.).

Tissue inflammation: Inflammation was expressed as the number of inflammatory cells per wall area (WA). Inflammatory cells included both granulocytes and mononuclear cells. The WA was defined as the area between the basement membrane and the outer aspect of the adventitial layer, defined by the alveolar attachments. Results were calibrated according to the magnification of the captured image. Values were obtained from two operators and correlations calculated. ($r^2 = 0.91$).

Mucus staining: Mucus was quantified by outlining the area of PAS positive staining within the epithelial area, using Image Pro Plus software. Results were expressed as the percent (%) mucus area divided by total epithelial area.

Airway smooth muscle: Basement membrane perimeter (P_{bm}) was measured as was the total area of α smooth muscle actin immunostaining (ASM) within the airway wall. Results were expressed as ASM divided by P_{bm} squared. Values were obtained from two operators and correlations calculated ($r^2 = 0.98$).

Collagen, decorin, and biglycan: Positive staining for collagen, decorin and biglycan within the WA was measured, based on previously determined stain-specific color settings (Image Pro Plus). Results were expressed as the area of positive stain/WA.

Central vs peripheral airways: Airways were sub-divided into central or peripheral according to basement membrane length. We calculated the median size of airways as 800 μ m, and defined those airways greater than the median as central, and those less than the median as peripheral.

2.10. Data analysis

Data are expressed as mean and standard error of the mean (SEM). Comparison of mechanical parameters and BAL cell differentials between saline and OVA-exposed mice was performed using one way analysis of variance (ANOVA) and Bonferoni's post-test. Comparisons of the OVA and saline groups with regards to number of inflammatory cells, mucus, airway smooth muscle, collagen and proteoglycans were made using 2 way ANOVA and Bonferoni's post-test, as were data comparing large and small airways. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Measurement of lung mechanics and responsiveness

Measurements of airway resistance (Raw), tissue damping (Gti) and tissue elastance (Hti) during methacholine-induced constriction are shown in Fig. 2. Data for the three saline (SAL) groups were pooled, as there were no significant differences among the three groups. Both the sub-acute (SA) and chronic (C) OVA groups showed significant increases in Raw as compared to saline (Fig. 2A). The SA group showed a significant increase with methacholine 12.5 and 25 mg/ml ($p < 0.01$ and 0.001 vs SAL, respectively) whereas the C group showed a significant increase in Raw with MCh 25 mg/ml,

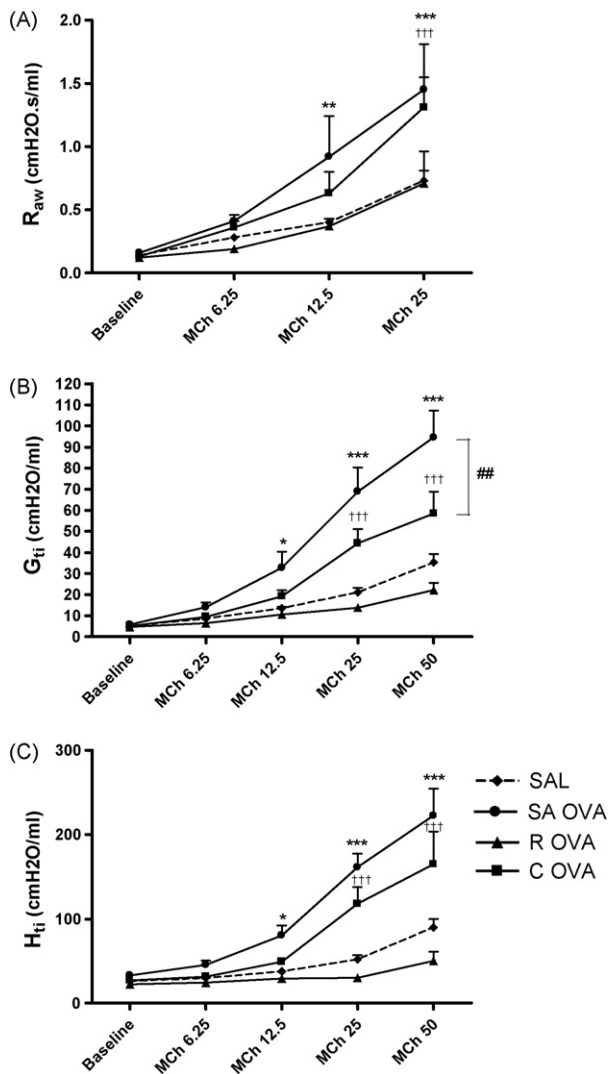


Fig. 2. Changes in airway resistance (R_{aw}), tissue damping (G_{ti}) and tissue elastance (H_{ti}) with delivery of methacholine (MCh) aerosol in the three ovalbumin challenged groups and saline control (A–C). (A) At the highest level of MCh (50 mg/ml), the sub-acute (SA) and chronic groups (C) showed an equivalent increase in R_{aw} as compared to the saline control; at the 25 mg/ml concentration, only the SA group showed a significant increase in R_{aw} ; the recovery group (46 days) showed a response that was not different from the saline group. (B) G_{ti} responsiveness was increased in both the SA and C groups compared to saline; however, the SA group showed a significant response at a lower concentration of MCh (12.5 mg/ml). Furthermore, the increase in the SA was significantly greater than the increase in the C group at the highest concentration of MCh. (C) Increases in H_{ti} responsiveness were significant for both the SA and C challenge groups; again the SA group showed a significant response at a lower concentration of MCh (12.5 mg/ml) than did the C group. * $p < 0.05$ vs saline control; ** $p < 0.01$ vs saline control; *** $p < 0.001$ vs saline control; ### $p < 0.01$ SA vs C.

only ($p < 0.001$ vs SAL). There were no differences in R_{aw} between the SA and C groups at all levels of MCh-induced airway constriction. The recovery (R) group was not different from SAL.

Measurement of tissue damping (G_{ti}) (Fig. 2B) showed that G_{ti} was increased in the SA group in response to MCh after delivery of 12.5 ($p < 0.05$); 25 ($p < 0.001$) and 50 ($p < 0.001$) mg/ml. The C group showed an increase in G_{ti} after MCh 25 and 50 mg/ml ($p < 0.001$). There was a significant difference in G_{ti} between the SA and C groups at the highest concentration of MCh ($p < 0.01$). The recovery (R) group was not different from SAL.

Tissue elastance (H_{ti}) was increased in the SA group in response to MCh after delivery of 12.5 ($p < 0.05$); 25 ($p < 0.001$) and 50 ($p < 0.001$) mg/ml (Fig. 2C). The C group showed an increase in H_{ti} after MCh 25 and 50 mg/ml ($p < 0.001$). There were no significant

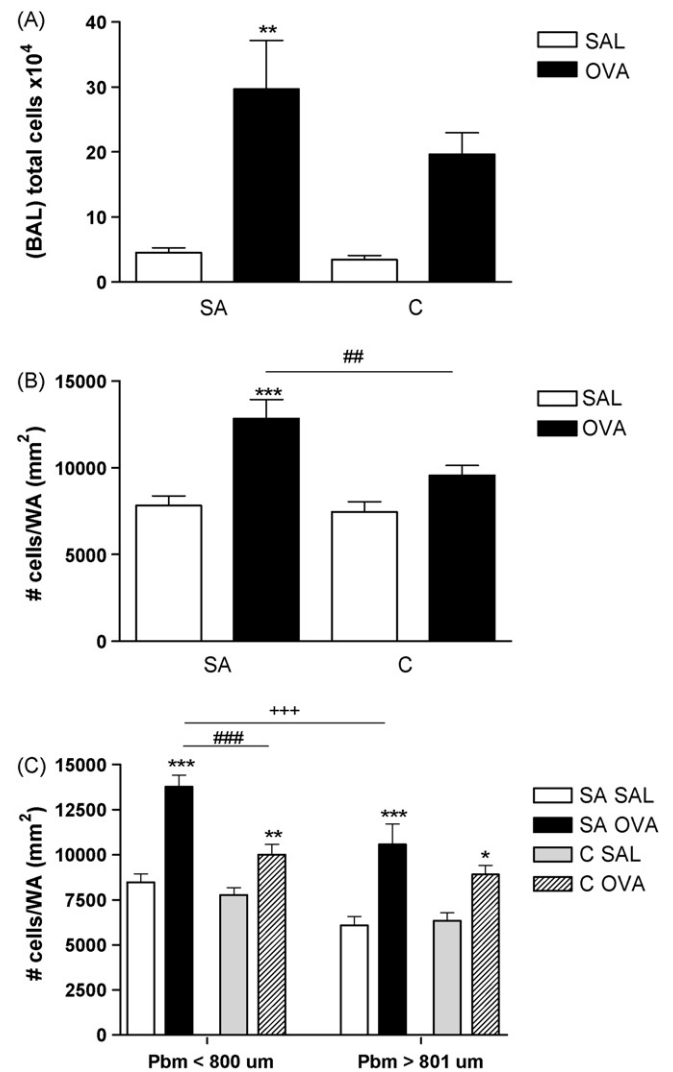


Fig. 3. (A) Total BAL cell count. Cell number was increased in the SA group only ($p < 0.001$ vs the respective saline control). (B) Tissue inflammation in the airway wall. The number of inflammatory cells per wall area (mm^2) was increased in the SA group as compared to the respective saline controls ($p < 0.001$). The increase in the SA group was greater than in the C group ($p < 0.01$). (C) Airways were divided into central and peripheral groups according to basement membrane perimeter. While both large and small airways showed increases in the number of inflammatory cells in both SA and C groups as compared to the respective saline controls, the greatest increase was in the SA small airways as compared to both the large airways and the C group. * $p < 0.05$ vs saline control; ** $p < 0.01$ vs saline control; *** $p < 0.001$ vs saline control; ## $p < 0.01$ vs SA; ++ $p < 0.01$ vs small airways.

differences in the H_{ti} response between the SA and C groups. The recovery (R) group was not different from SAL.

3.2. Indices of inflammation

Bronchoalveolar lavage (BAL) results are reported in Fig. 3A. The total cell number was increased after SA challenge ($p < 0.001$ vs the respective SAL) and returned to control levels after a period of recovery (R). The increase in cell number in C OVA did not attain statistical significance. The cell differential, however, showed a similar increase in eosinophils with OVA, in the SA and C groups ($67.3 \pm 3.9\%$ and $54.8 \pm 5.6\%$, respectively). For the R group, the cell differential resembled that of the saline control (data not shown).

Photomicrographs of tissue inflammation in the airway wall are shown in Fig. 4a–c, and the number of inflammatory cells/WA quantified in Fig. 3B and C. There was a significant increase in the

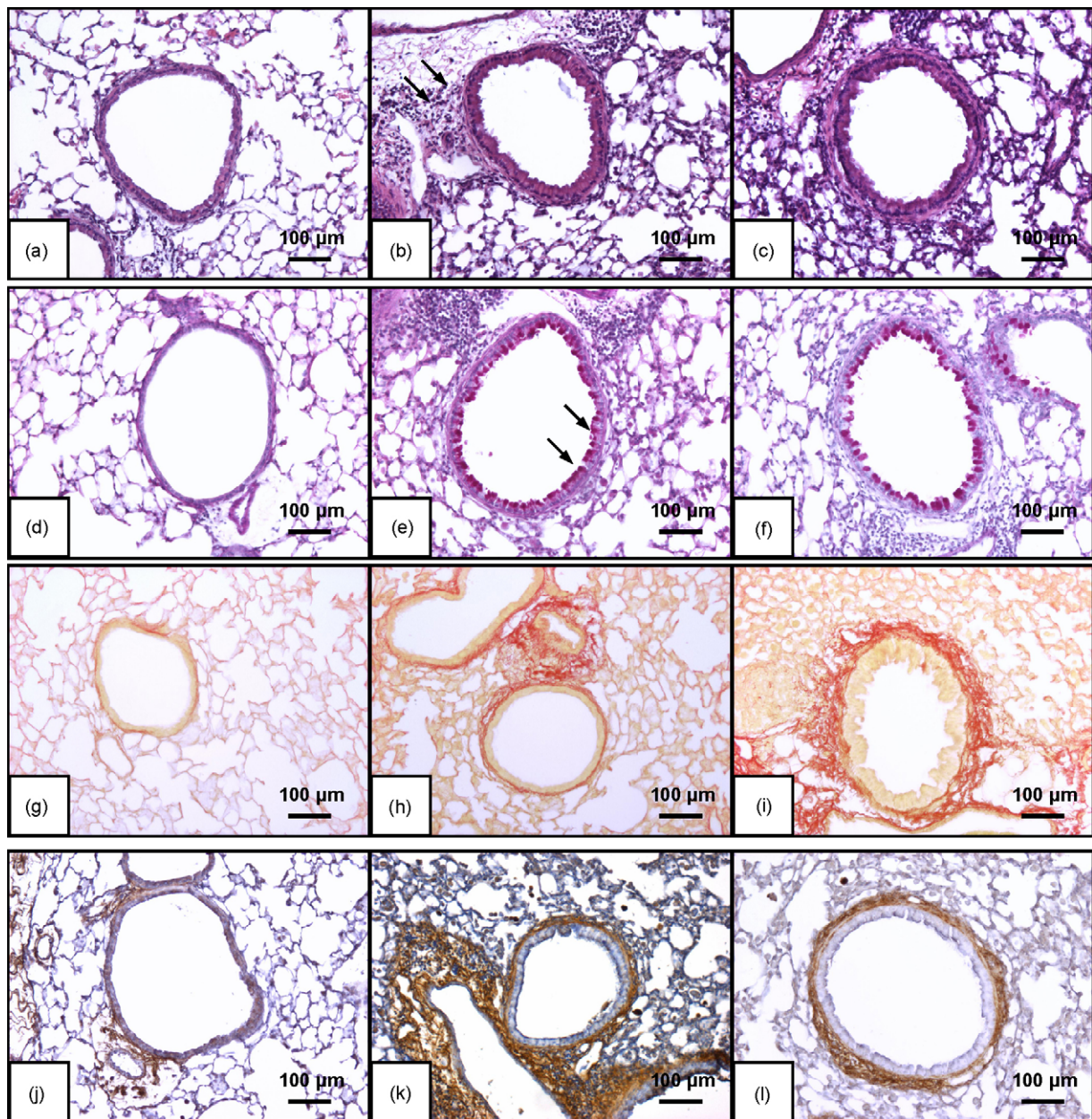


Fig. 4. Photomicrographs of tissue inflammation (H&E) (a–c); mucus (PAS) (d–f); collagen (picosirius red) (g–i); and decorin immunostaining (j–l). Left hand panels are SAL airways; middle panels are SA OVA airways; right hand panels are C OVA airways. Arrows in panel (b) identify inflammatory cells. Arrows in panel (e) highlight mucus staining. All images are taken at 10 $\times$ magnification.

number of cells after SA challenge ($p < 0.001$). The number of cells was greater in the SA vs C group ($p < 0.01$). The increase in inflammatory cell number was further examined to determine whether this was a central or distal airway response. There were significant increases in inflammatory cells in large and small airways in both SA and C groups vs their respective saline controls. The relative increase was greatest in small vs large airways in the SA group ($p < 0.01$) and in the small airways in the SA vs C groups ($p < 0.01$).

3.3. Airway mucus

Airway mucus expression is shown in representative airways in Fig. 4d–f. The area of PAS positive staining, was increased in both the SA and C OVA groups as compared to SAL (Fig. 5A). There was no difference between these SA and C OVA. PAS staining was increased

in both large and small airways after SA and C OVA (Fig. 5B). The increase in mucus in the peripheral airways was greater in the SA vs C challenge group, whereas, in the central airways, the increase was greater in C OVA. In C OVA, the increase in mucus staining was greater in large vs small airways. In the R group, airway mucus expression resembled that of the saline control (data not shown).

3.4. Airway smooth muscle

Airway smooth muscle was identified by immunostaining. While there was a trend towards an increase in the area of ASM/Pbm² with OVA challenge when compared to SAL, in both SA and C groups, these changes did not reach statistical significance [$2.57 \pm 0.47 \times 10^3$ vs $1.73 \pm 0.44 \times 10^3$ (SA OVA vs SA SAL) and $2.29 \pm 0.25 \times 10^3$ vs $1.48 \pm 0.26 \times 10^3$ (C OVA vs C SAL)].

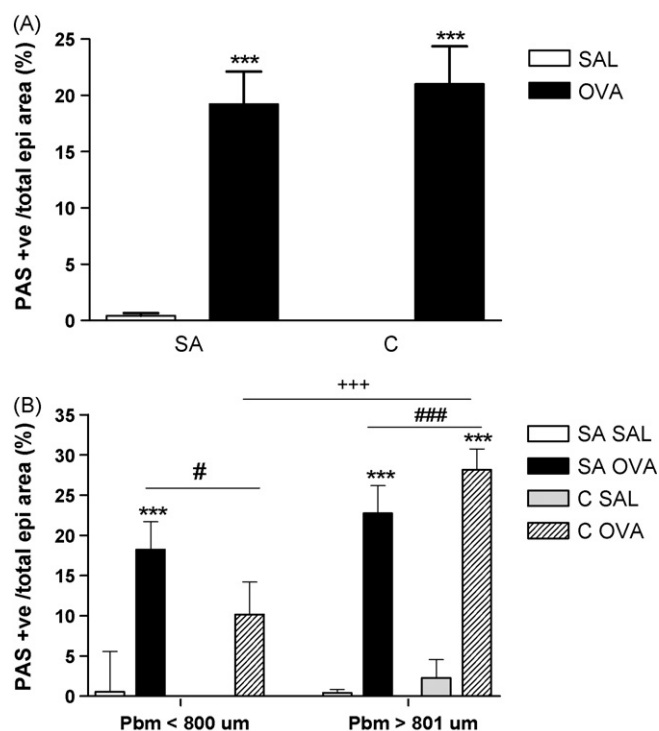


Fig. 5. Airway mucus in the epithelial layer as measured by PAS positive staining. (A) The % PAS positive staining within the epithelial layer per total epithelial area was increased in both SA and C OVA groups as compared to their respective saline controls ($p < 0.001$). The increase in the SA and C group was similar. (B) Airways were divided into central and peripheral groups according to basement membrane perimeter. Both large and small airways showed increases in the area of mucus in both SA and COVA groups as compared to saline. In the SA OVA, the increase in mucus in the small airways was greater than in the C OVA; in the C OVA, the increase in the large airways was greater than that in the SA OVA and in the C OVA small airways ($p < 0.001$). * $p < 0.05$ vs saline control; ** $p < 0.01$ vs saline control; *** $p < 0.001$ vs saline control; *** $p < 0.001$ vs small airways.

3.5. Extracellular matrix

To assess remodelling of the extracellular matrix, changes in collagen, biglycan and decorin deposition were quantified. Collagen deposition is shown in selected airways in Fig. 4g–i. In both the SA and C groups, there was an increase in the area of collagen as compared to the respective SAL (Fig. 6A). The increase in collagen in SA OVA was evident in both central and peripheral airways; the increase in C OVA was present in central airways, only (Fig. 6B). In the R group, the area of collagen was equivalent to that of SAL (data not shown). Biglycan deposition in the airway wall was increased in both SA and C OVA (Fig. 7A). The increased deposition was significant in both the central and peripheral airways in the C group and in the central airways only in the SA group (Fig. 7B). Decorin immunostaining was significantly increased after both SA and C OVA (Figs. 4j–l and 7C); again, in C OVA, staining was increased in both central and peripheral airways. In SA OVA, decorin staining was increased in the peripheral airways, only (Fig. 7D).

4. Discussion

In this study, we show that both sub-acute and chronic ovalbumin challenge in C57Bl/6 mice lead to significant airway hyper-responsiveness, evident at both the levels of the central airways and more distal lung. In the sub-acute model, distal airway hyper-responsiveness occurred to a greater degree. Inflammation was present after both sub-acute and chronic challenge, but more marked after sub-acute challenge, particularly in the distal airways.

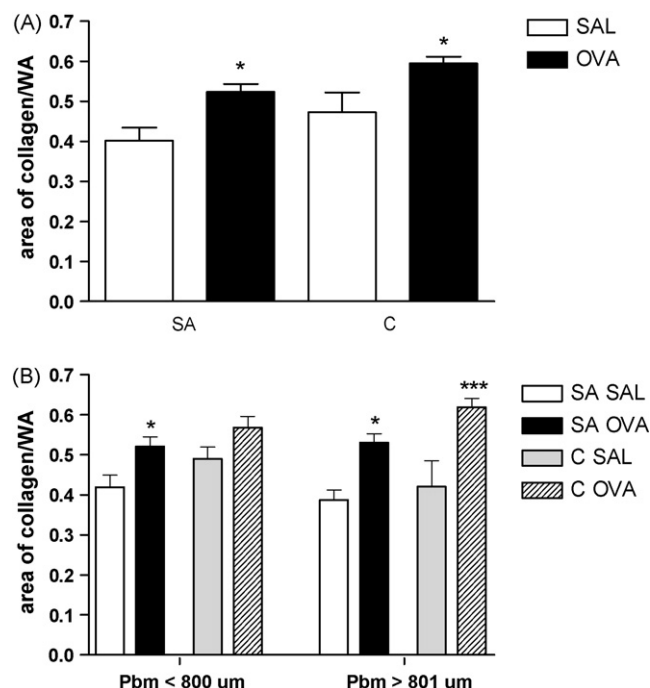


Fig. 6. Staining for collagen using picrosirius red. (A) The area of positive staining for collagen per airway wall area was increased compared to the respective saline controls in both SA and COVA groups as compared to their respective saline controls ($p < 0.05$). (B) Airways were divided into central and peripheral according to basement membrane perimeter. In the SA OVA group, collagen staining was increased in both small and large airways as compared to their respective saline controls. In C OVA, only the large airways showed an increase in the area of collagen ($p < 0.001$). ** $p < 0.01$ vs saline control; # $p < 0.05$ vs SA.

Airway mucus was similarly enhanced. Airway remodelling, specifically increases in extracellular matrix, occurred to an equivalent degree, after both sub-acute and chronic challenge. In this mouse model, relative differences in peripheral vs central airway hyper-responsiveness, seem to be related to differences in the degree of inflammation, rather than remodelling, per se.

A number of investigators have used allergen challenge in murine models in an attempt to dissect the mechanisms driving the various components of the asthmatic response (Shinagawa and Kojima, 2003; Hirota et al., 2006; Southam et al., 2007; Locke et al., 2007). Whereas hyper-responsiveness has been specifically linked to inflammation in some studies (Lee et al., 2004), other studies have defined airway remodelling as an important contributor (Leigh et al., 2002; Locke et al., 2007). One of the issues that have been identified, is the duration of allergen challenge. It has been suggested that after acute challenge, inflammation drives hyper-responsiveness, whereas remodelling becomes a more critical mechanism after chronic challenge (Leigh et al., 2002; Southam et al., 2007). The data of the current experiment refute this argument somewhat, at least in C57Bl/6 mice. We found that the degree of remodelling was essentially equivalent after both sub-acute and chronic challenge. Conversely, inflammation was greater after sub-acute challenge. This increased inflammation was associated with a relatively greater degree of hyper-responsiveness.

Responsiveness was further characterized by partitioning physiologic changes into central and peripheral lung components. Complex impedance was measured, and changes in mechanics were separated into central airway resistance and the peripheral indices, tissue damping and elastance. The latter reflect responses in the peripheral airways and parenchymal tissues (Hantos et al., 1992; Lutchen et al., 1996). Under control conditions, tissue damping reflects energy dissipation in the lung tissues or tissue viscance,

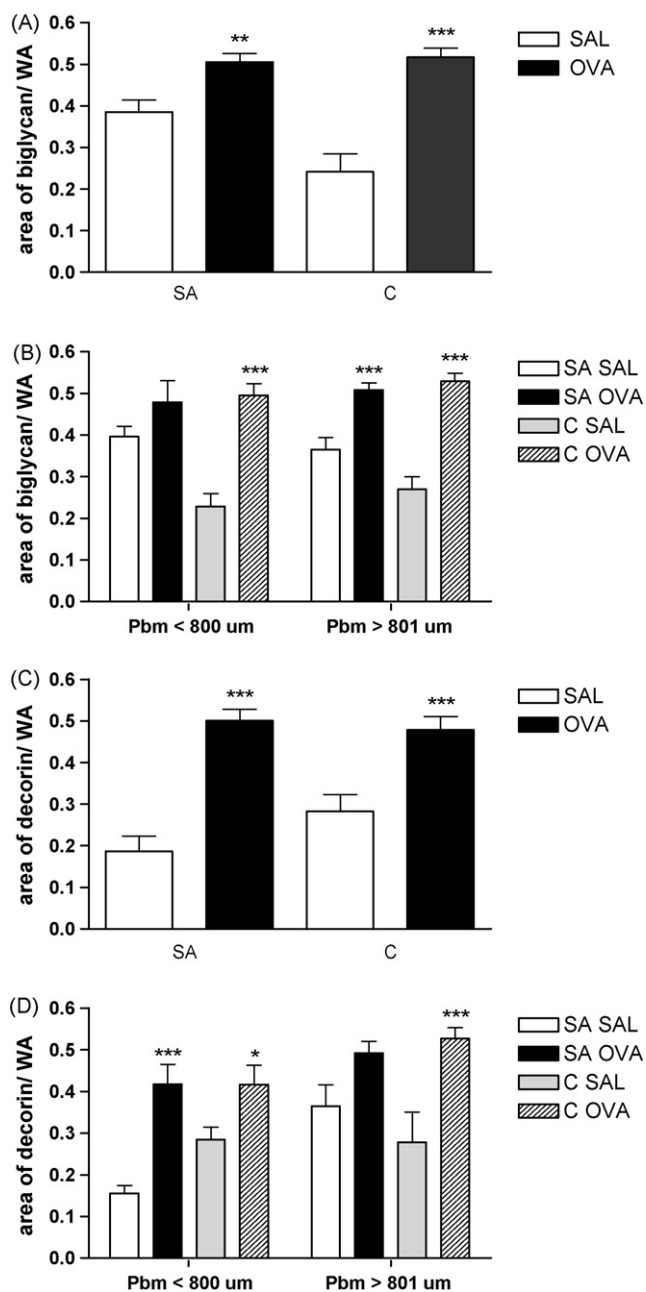


Fig. 7. Biglycan and decorin immunostaining. (A) The area of positive staining for biglycan per airway wall area was increased compared to the respective saline control in both SA and C OVA ($p < 0.01$ and 0.001 , respectively). (B) Airways were divided into central and peripheral groups according to basement membrane perimeter. In the SA OVA group, biglycan staining was significantly increased in the large airways, only. In the C OVA group, both the large and small airways showed an increase in the area of biglycan ($p < 0.001$). ** $p < 0.01$ vs saline control; *** $p < 0.01$ vs saline control. (C) The area of positive staining for decorin per airway wall area was increased in both SA and C OVA compared to the respective saline controls ($p < 0.001$). (D) Airways were divided into central and peripheral groups according to basement membrane perimeter. In SA OVA, decorin staining was significantly increased in the small airways only. In C OVA, both the large and small airways showed an increase in the area of decorin. * $p < 0.05$ vs saline control; ** $p < 0.01$ vs saline control; *** $p < 0.01$ vs saline control.

and tissue elastance reflects energy storage. During induced constriction, increases in tissue damping (Gti) are increasingly related to peripheral airway heterogeneities and constriction-induced airway closure, and hence, become more reflective of small airway constriction (Kaczka et al., 1997; Ito et al., 2007). Asthma is a disease characterized by heterogeneous airway constriction, and it

is generally accepted that much of this heterogeneous response occurs in the distal lung (Hamid et al., 1997; Kraft, 1999). Therefore, defining the contribution of central vs peripheral airways becomes increasingly important, especially when trying to understand relations among changes in responsiveness and changes in indices such as inflammation, airway mucus, airway smooth muscle and airway remodelling. Other investigators have shown that remodelling in asthma does not occur in a uniform manner throughout the airway tree, whether human asthma or animal models are studied (de Medeiros et al., 2005; Bergeron et al., 2005; Pini et al., 2006; Hirota et al., 2006). The data of the current experiment give insight into the mechanisms driving the changes in responsiveness at the different levels of the airway tree.

Increases in central airway responsiveness were similar after sub-acute and chronic ovalbumin exposures. On the other hand, increases in distal responsiveness were more significant in the sub-acute model. Significant increases in tissue damping and elastance occurred at lesser concentrations of methacholine in the sub-acute model and the increase in damping at the highest concentration of methacholine was significantly greater in the SA vs C OVA group. These differences in physiology were associated with differences in inflammation. BAL total cell number was greater in SA vs C OVA; further, the increase in the number of airway inflammatory cells was greater in SA OVA. This difference was largely accounted for by differences found in the number of cells populating airways with Pbm < 800 μ m. Another potential contributor to this difference in airway physiology is airway mucus. PAS positive staining was significantly greater in the peripheral airways of the SA vs C OVA group. Excess airway mucus may contribute to narrowing of the airway lumen and subsequent hyper-responsiveness. The association between inflammatory mediators, such as IL-4 and IL-9, and mucus hypersecretion has been previously reported (Temann et al., 1997; Toda et al., 2002).

Surprisingly, the degree of remodelling was not different after sub-acute and chronic challenge. While there were modest increases in airway smooth muscle, these changes did not reach statistical significance. Collagen deposition was increased after both sub-acute and chronic challenge (in C OVA, the increase vs saline was evident in the large airways, only). However, there were no significant differences between SA and C OVA. Similar findings were observed for the two proteoglycans sampled, decorin and biglycan. This lack of enhanced remodelling after more chronic challenge is distinct from the pattern previously reported (Southam et al., 2007; Locke et al., 2007), but may be attributable to differences in the strain examined, or the precise protocol of the allergen challenge. The similar remodelling observed in the current experiment, may explain the similarities in the central airway responsiveness in the SA and C OVA mice.

The apparent disconnection between inflammation and remodelling is worth considering further. The usual paradigm of inflammatory cell influx with release of pro-inflammatory and pro-remodelling cytokines leading to airway remodelling (Locke et al., 2007), does not seem to be operative in the C57Bl/6 model studied here. Inflammation was greater in the SA model, although the relative proportion of eosinophils in the BAL was similar in SA and C OVA. One might have anticipated a greater degree of remodelling in the SA OVA mice, given the greater degree of inflammation. One could postulate that there was insufficient time for the remodelling to fully develop in these mice, but the results in the recovery group argue to the contrary. In the R group, airway hyper-responsiveness reverted to control values, as did the indices of inflammation, mucus and remodelling. The ability of these mice to recover to their native state is worth remarking, and suggests that it is not simply a lack of adequate time for the remodelling to occur, which explains the differences in hyper-responsiveness in the SA and C OVA mice.

C57Bl/6 mice are a common background for genetic manipulation; defining the response of this mouse strain to allergen sensitization and challenge is of particular interest. Strain-related differences in response to allergen challenge is well described (Wills-Karp and Ewart, 1997; Ewart et al., 2000; Shinagawa and Kojima, 2003; An et al., 2007), and the strain of mouse chosen for transgenic or knockout studies is “likely to impact the results” (Shapiro, 2006). The data of the current study underline differences in the response of C57Bl/6 mice, as compared to that observed in other strains, such as BALB/c, or A/J. Insofar as murine models of asthma provide a valuable tool, defining a precise protocol whereby the C57Bl/6 mouse responds to ovalbumin challenge, and characterizing the specifics of that response, is important for the study of mechanisms underlying the development of asthmatic disease (Shapiro, 2006).

5. Conclusion

The present study highlights the contribution of inflammation, mucus production and various indices of remodelling in both the central and peripheral airways of C57Bl/6 mice exposed to a sub-acute and a chronic challenge protocol. In particular, the data suggest that inflammation and airway mucus in the lung periphery drive the mechanical response in the sub-acute challenge model. Moreover, the presence of airway remodelling, which occurred to a similar extent in both sub-acute and chronic challenge models, may be a contributing factor accounting for the similar increases in central airway responsiveness between these experimental groups. This study also defines and characterizes an ovalbumin challenge protocol in a murine strain that is extensively used for knockout or over-expression studies.

Contributors

VP helped design and conducted most of the experiments, performed the data analysis, and helped write the manuscript. CLM helped design, conduct the experiments, performed data analysis, and helped write the manuscript. MSL helped design and analyze the experiments, and write the manuscript.

Acknowledgements

This study was funded and supported by the J T Costello Memorial Research Fund, the Canadian Institutes of Health Research, a center grant from the Fonds de la Recherche en Santé du Québec and the McGill University Health Center Research Institute (CLM).

References

- Alcorn, J.F., Rinaldi, L.M., Jaffe, E.F., van, L.M., Bates, J.H., Janssen-Heininger, Y.M., Irvin, C.G., 2007. Transforming growth factor-beta1 suppresses airway hyperresponsiveness in allergic airway disease. *Am. J. Respir. Crit. Care Med.* 176, 974–982.
- An, S.S., Bai, T.R., Bates, J.H., Black, J.L., Brown, R.H., Brusasco, V., Chitano, P., Deng, L., Dowell, M., Eidelman, D.H., Fabry, B., Fairbank, N.J., Ford, L.E., Fredberg, J.J., Gerthoffer, W.T., Gilbert, S.H., Gosens, R., Gunst, S.J., Halayko, A.J., Ingram, R.H., Irvin, C.G., James, A.L., Janssen, L.J., King, G.G., Knight, D.A., Lauzon, A.M., Lakser, O.J., Ludwig, M.S., Lutchen, K.R., Maksym, G.N., Martin, J.G., Mauad, T., McParland, B.E., Mijailovich, S.M., Mitchell, H.W., Mitchell, R.W., Mitzner, W., Murphy, T.M., Pare, P.D., Pellegrino, R., Sanderson, M.J., Schellenberg, R.R., Seow, C.Y., Silveira, P.S., Smith, P.G., Solway, J., Stephens, N.L., Sterk, P.J., Stewart, A.G., Tang, D.D., Tepper, R.S., Tran, T., Wang, L., 2007. Airway smooth muscle dynamics: a common pathway of airway obstruction in asthma. *Eur. Respir. J.* 29, 834–860.
- Bergeron, C., Hauber, H.P., Gotfried, M., Newman, K., Dhanda, R., Servi, R.J., Ludwig, M.S., Hamid, Q., 2005. Evidence of remodeling in peripheral airways of patients with mild to moderate asthma: effect of hydrofluoroalkane-flunisolide. *J. Allergy Clin. Immunol.* 116, 983–989.
- de Medeiros, M.M., da Silva, L.F., dos Santos, M.A., Ferneznian, S., Schrupf, J.A., Roughley, P., Hiemstra, P.S., Saldiva, P.H., Mauad, T., Dolnikoff, M., 2005. Airway proteoglycans are differentially altered in fatal asthma. *J. Pathol.* 207, 102–110.
- Ewart, S.L., Kuperman, D., Schadt, E., Tankersley, C., Grupe, A., Shubitski, D.M., Peltz, G., Wills-Karp, M., 2000. Quantitative trait loci controlling allergen-induced airway hyperresponsiveness in inbred mice. *Am. J. Respir. Cell Mol. Biol.* 23, 537–545.
- Hamid, Q., Song, Y., Kotsimbos, T.C., Minshall, E., Bai, T.R., Hegele, R.G., Hogg, J.C., 1997. Inflammation of small airways in asthma. *J. Allergy Clin. Immunol.* 100, 44–51.
- Hantos, Z., Daroczy, B., Suki, B., Nagy, S., Fredberg, J.J., 1992. Input impedance and peripheral inhomogeneity of dog lungs. *J. Appl. Physiol.* 72, 168–178.
- Hirota, J.A., Ellis, R., Inman, M.D., 2006. Regional differences in the pattern of airway remodeling following chronic allergen exposure in mice. *Respir. Res.* 7, 120.
- Ito, S., Lutchen, K.R., Suki, B., 2007. Effects of heterogeneities on the partitioning of airway and tissue properties in normal mice. *J. Appl. Physiol.* 102, 859–869.
- Kaczka, D.W., Ingenito, E.P., Suki, B., Lutchen, K.R., 1997. Partitioning airway and lung tissue resistances in humans: effects of bronchoconstriction. *J. Appl. Physiol.* 82, 1531–1541.
- Kraft, M., 1999. The distal airways: are they important in asthma? *Eur. Respir. J.* 14, 1403–1417.
- Lee, J.J., Dimina, D., Macias, M.P., Ochkur, S.I., McGarry, M.P., O'Neill, K.R., Protheroe, C., Pero, R., Nguyen, T., Cormier, S.A., Lenkiewicz, E., Colbert, D., Rinaldi, L., Ackerman, S.J., Irvin, C.G., Lee, N.A., 2004. Defining a link with asthma in mice congenitally deficient in eosinophils. *Science* 305, 1773–1776.
- Leigh, R., Ellis, R., Wattie, J., Southam, D.S., De, H.M., Gauldie, J., O'Byrne, P.M., Inman, M.D., 2002. Dysfunction and remodeling of the mouse airway persist after resolution of acute allergen-induced airway inflammation. *Am. J. Respir. Cell Mol. Biol.* 27, 526–535.
- Locke, N.R., Royce, S.G., Wainwright, J.S., Samuel, C.S., Tang, M.L., 2007. Comparison of airway remodeling in acute, subacute, and chronic models of allergic airways disease. *Am. J. Respir. Cell Mol. Biol.* 36, 625–632.
- Lutchen, K.R., Hantos, Z., Petak, F., Adamczak, A., Suki, B., 1996. Airway inhomogeneities contribute to apparent lung tissue mechanics during constriction. *J. Appl. Physiol.* 80, 1841–1849.
- Pini, L., Torregiani, C., Martin, J.G., Hamid, Q., Ludwig, M.S., 2006. Airway remodeling in allergen-challenged Brown Norway rats: distribution of proteoglycans. *Am. J. Physiol. Lung Cell Mol. Physiol.* 290, L1052–L1058.
- Shapiro, S.D., 2006. Animal models of asthma: Pro: allergic avoidance of animal (model[s]) is not an option. *Am. J. Respir. Crit. Care Med.* 174, 1171–1173.
- Shinagawa, K., Kojima, M., 2003. Mouse model of airway remodeling: strain differences. *Am. J. Respir. Crit. Care Med.* 168, 959–967.
- Southam, D.S., Ellis, R., Wattie, J., Inman, M.D., 2007. Components of airway hyperresponsiveness and their associations with inflammation and remodeling in mice. *J. Allergy Clin. Immunol.* 119, 848–854.
- Temann, U.A., Prasad, B., Gallup, M.W., Basbaum, C., Ho, S.B., Flavell, R.A., Rankin, J.A., 1997. A novel role for murine IL-4 in vivo: induction of MUC5AC gene expression and mucin hypersecretion. *Am. J. Respir. Cell Mol. Biol.* 16, 471–478.
- Toda, M., Tulic, M.K., Levitt, R.C., Hamid, Q., 2002. A calcium-activated chloride channel (HCLCA1) is strongly related to IL-9 expression and mucus production in bronchial epithelium of patients with asthma. *J. Allergy Clin. Immunol.* 109, 246–250.
- Whitehead, G.S., Walker, J.K., Berman, K.G., Foster, W.M., Schwartz, D.A., 2003. Allergen-induced airway disease is mouse strain dependent. *Am. J. Physiol. Lung Cell Mol. Physiol.* 285, L32–L42.
- Wills-Karp, M., Ewart, S.L., 1997. The genetics of allergen-induced airway hyperresponsiveness in mice. *Am. J. Respir. Crit. Care Med.* 156, S89–S96.