

Hypertrophic Airway Smooth Muscle Mass Correlates with Increased Airway Responsiveness in a Murine Model of Asthma

Pamela J. Plant^{1,3}, Michelle L. North^{1,2}, Andrew Ward³, Michael Ward^{1,3†}, Nivedita Khanna^{1,2}, Judy Correa^{1,3}, Jeremy A. Scott^{1,2,3*}, and Jane Batt^{1,3*}

¹Division of Clinical Sciences, Department of Medicine, and ²Gage Occupational and Environmental Health Unit, Dalla Lana School of Public Health and Department of Medicine, University of Toronto, Toronto, Ontario, Canada; and ³Keenan Research Centre, Li Ka Shing Knowledge Institute, St Michael's Hospital, Toronto, Ontario, Canada

The increase of airway smooth muscle (ASM) mass in asthma results from hypertrophic and hyperplastic stimuli, and leads to an increase in cellular contractile proteins. However, little evidence correlates the relative contributions of hypertrophic and hyperplastic muscle with functional effects on airway resistance. We performed a ventilator-based assessment of respiratory mechanics and responsiveness to methacholine in a murine model of acute (3-week) ovalbumin (OVA)-induced airway inflammation, compared with a chronic (12-week) model. We correlated functional changes in airways Newtonian resistance (R_N), peripheral tissue damping (G), and elastance (H) with the relative contributions of proliferation, hypertrophy, and apoptosis to increased ASM mass. Immunohistochemical analyses of treated (OVA-sensitized and OVA-challenged; OVA/OVA) and control (OVA-sensitized and saline-challenged; OVA/PBS) murine lungs showed an increase in ASM area in chronic, but not acute, OVA/OVA-treated mice that correlated positively with increased airway resistance to methacholine. Acute OVA/OVA-treated ASM exhibited an increase in proliferation with diminished apoptosis, which resolved in the chronic OVA/OVA model. Chronic OVA/OVA-treated ASM exhibited hypertrophy. Distinct temporal differences exist in the response of murine airways to antigenic challenge. We report that ASM proliferation and diminished apoptosis occur during the acute phase, followed by the development of smooth muscle hypertrophy and an increased muscle mass with chronic challenge, that correlate strongly with increased airway Newtonian resistance. The identification of a functionally relevant hypertrophic bronchial muscle mass highlights the possibility of regulating airway muscle hypertrophy as a novel therapeutic target in asthma.

Keywords: hypertrophy; proliferation; apoptosis; constant-phase model; airway smooth muscle

Abnormal airway reactivity, which can be exacerbated by structural remodeling, contributes to the pathophysiology of many lung diseases, including asthma. Inflammation of the airways is implicated as a causative factor underlying the changes in airway structure that

CLINICAL RELEVANCE

We demonstrate that the proliferative and hypertrophic responses of airway smooth muscle occur in temporally distinct, sequential phases in murine models of allergen-induced airway inflammation. The hypertrophic muscle mass is the predominant feature of the remodeled airway that correlates with airway hyperreactivity, and it could constitute an important therapeutic target in the treatment of asthma.

accompany disease progression (1–5). Typically these changes include mucosal thickening, inflammatory cell and fibroblast infiltration, epithelial hyperplasia, and goblet-cell metaplasia. Increased airway smooth muscle cell (ASMC) mass, resulting from hypertrophy (increased ASMC size) and hyperplasia (increased ASMC numbers, attributable to increased proliferation and/or diminished apoptosis), is also observed (6–8).

Several functional consequences of the increased smooth muscle mass that may underlie the pathophysiology of asthma have been proposed. Mathematical models reveal that the increased muscle mass is likely the most important abnormality responsible for the increased resistance observed in response to bronchoconstricting stimuli in patients with asthma (9). The increased muscle mass resulting from allergen sensitization and repeated challenges is associated with airway hyperresponsiveness in murine models *in vivo* (10–12). In tissue culture models of bronchial smooth muscle hypertrophy induced by transforming growth factor- β 1, cardiotropin-1, or LiCl, increases in smooth muscle α -actin content correlate with increased cell shortening in response to acetylcholine and KCl (13, 14). The effect of ASMC proliferation on contraction is not as clear-cut. Uncoupling of the proliferative response and smooth muscle contractility has been demonstrated and appears to result from a transition of proliferating ASMCs to a synthetic phenotype expressing fetal contractile protein isoforms with a diminished contractile capacity and lower contractile protein content (15). In contrast, other studies demonstrated the enhanced *in vitro* proliferative capacity and hypercontractility of ASMCs derived from patients with asthma (16–18).

Both smooth muscle hypertrophy and hyperplasia were observed to increase the airway muscle mass in human asthma, and the variations in the relative proportions reported seem to depend in some part upon disease severity, disease duration, and the generation airways assessed (6–8, 19). For example, the morphometric analysis of endobronchial biopsies from patients with differing severities of asthma revealed airway smooth muscle hypertrophy and fibroblast accumulation to be selective determinants of severe, persistent asthma (6). Subjects with mild

(Received in original form August 23, 2011 and in final form November 17, 2011)

* These authors contributed equally to this work.

† Michael Ward is deceased.

This study was supported by operating grants from the Canadian Institutes of Health Research (J.B. and M.W.), a Keenan Centre Summer Student Research Award (A.W.), AllerGen Networks of Centres of Excellence and the National Sanatorium Association (J.A.S.), and Canadian Institutes of Health/Ontario Thoracic Society Doctoral Awards (M.L.N.).

Correspondence and requests for reprints should be addressed to Jane Batt, M.D., Ph.D., Keenan Research Centre, Li Ka Shing Knowledge Institute, St. Michael's Hospital, 209 Victoria St., Room 616, Toronto, ON, M5B 1T8 Canada. E-mail: jane.batt@utoronto.ca

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 46, Iss. 4, pp 532–540, Apr 2012

Copyright © 2012 by the American Thoracic Society

Originally Published in Press as DOI: 10.1165/rcmb.2011-0293OC on November 22, 2011

Internet address: www.atsjournals.org

to moderate asthma showed a smooth muscle cell size similar to that of their control counterparts, but with nearly double the number of cells (7). Despite the widespread knowledge that both increased cell number and increased cell size/contractile protein content contribute to the increased muscle content of asthmatic airways, the relative contributions of these two processes to altered pulmonary mechanics *in vivo* and their relative distribution in the asthmatic airway remain uncertain. Accordingly, the purpose of the present study was to determine the relative contributions of airway smooth muscle hypertrophy, muscle cell proliferation, and diminished apoptosis to airway hyperresponsiveness in acute and chronic murine models of OVA-induced allergic airway inflammation. Furthermore, we sought to perform correlative analyses between these morphological changes and the *in vivo* physiological responsiveness of the airways, to reveal the relationship between impaired function and the increased smooth muscle mass induced by cellular hypertrophy, cellular proliferation, or reduced cell loss through the inhibition of apoptosis.

MATERIALS AND METHODS

Please see the online supplement for detailed experimental protocols.

Acute and Chronic Ovalbumin-Sensitization and Challenge Models

We used two well-established models of allergic airway inflammation to ovalbumin (OVA) (20). Female BALB/c mice were sensitized to chicken OVA (intraperitoneal), and randomized to repeated exposure to nebulized 2.5% OVA or PBS for 3 weeks (acute model) or 12 weeks (chronic model). Twenty-four hours after the final OVA or PBS challenge, the mice were anesthetized and intubated for an *in vivo*, ventilator-based assessment of methacholine responsiveness, using flexiVent (Scireq, Inc., Montreal, PQ, Canada). We determined the Newtonian resistance of the airways (R_N) and peripheral tissue damping and elastance (G and H, respectively) (21–25). After measurement of the airways responsiveness to methacholine, the mice were killed and their lungs were isolated and inflation-fixed in formalin.

Tissue Preparation, Immunohistochemistry, Immunofluorescence and Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick-End Label Assay

Isolated lungs were cut into 5- μ m sections, and airways were selected for histologic assessment according to established criteria (26). Serial sections were used for immunohistochemistry, immunofluorescence, and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end label (TUNEL) staining, to determine airway smooth muscle area and identify proliferating and apoptotic nuclei, respectively.

Morphometric Assessment of Airway Smooth Muscle Mass and Hypertrophic, Proliferation, and TUNEL Indices

Morphometric analysis of airway area and smooth muscle area was performed with ImageJ software (National Institutes of Health, Bethesda, MD). Smooth muscle area was normalized to airway basement membrane length in each airway for comparative analyses and correlations. Airways were also stratified into small (< 50,000 μ m²), intermediate (50,000–175,000 μ m²), and large (> 175,000 μ m²), based on the airway area internal to the epithelial basement membrane, as previously defined (26), for subsequent analyses and correlations.

To determine myocyte size and the extent of proliferation, immunofluorescently stained sections were visualized, and total nuclei and Ki67-positive (actively proliferating) nuclei within the smooth muscle were counted in areas that stained positive for smooth muscle actin. The smooth muscle area was expressed per cell (i.e., airway muscle area/total number of smooth muscle nuclei) and normalized to airway basement membrane

length as an index of hypertrophy (hypertrophic index). Similarly, the number of proliferating nuclei (assessed by Ki67-positive staining) was expressed as a ratio of total smooth muscle nuclei and normalized to airway basement membrane length (proliferation index). TUNEL-positive nuclei were scored by three independent observers in a blinded manner. Scores were expressed as the ratio of TUNEL-positive nuclei to total nuclei in the airway smooth muscle, normalized to basement membrane length.

Statistical Analyses

Data are expressed as means $\pm$ SEM. Methacholine dose-response curves were compared using the F-test. Two-way ANOVA and the Student *t* test were used to compare measures between groups, where appropriate. For all normalized morphometric data, the mean of all airway measurements per mouse was calculated at each time point (3 weeks or 12 weeks), and comparisons between OVA/OVA-treated and OVA/PBS-treated mice were undertaken using the Student *t* test. For comparisons across the predefined size-stratified airway cohorts (small, medium, and large), data were assessed by two-way ANOVA. Statistical significance was assumed in all cases if $P < 0.05$. Associations between functional parameters (i.e., R_N , G, and H) and morphometric endpoints were made using the Spearman correlation, and these associations were two-tailed.

RESULTS

OVA Sensitization and Challenge Increases Airway Responsiveness to Methacholine

Methacholine (MCh; 0.01–100 mg/ml) was nebulized directly into the ventilatory circuit to assess *in vivo* airway responsiveness (Figure 1). The R_N of the airways was significantly augmented in the OVA-sensitized and OVA-challenged (OVA/OVA) mice in both the acute (Figure 1A) and chronic (Figure 1B) models, compared with the respective OVA-sensitized and PBS-challenged controls (OVA/PBS). Peripheral tissue damping (Figures 1C and 1D) and elastance (Figures 1E and 1F) were similarly augmented.

Airway Smooth Muscle Mass Is Increased in the Chronic (12-Week) Model of Ovalbumin-Induced Allergic Asthma

Hematoxylin and eosin staining revealed a robust inflammatory reaction centered on the bronchovascular bundles in the OVA-sensitized and OVA-challenged mice from the acute 3-week model, which had resolved substantially by 12 weeks in the chronic model (Figure 2A). To identify and quantify the muscle content of airways, we immunostained lung sections for α -smooth muscle actin (Figure 2B), measured the area of bronchial smooth muscle, and normalized it to basement membrane length (Figures 2C and 2D). In the chronic 12-week model, a significant increase was evident in the smooth muscle area of all airways in the OVA-sensitized and OVA-challenged mice ($7.72 \pm 0.52 \mu$ m²/ μ m) versus saline-challenged mice ($5.36 \pm 0.28 \mu$ m²/ μ m; $P < 0.05$; Figure 2C, *right*). Further analysis revealed no regional variation throughout the bronchial tree, insofar as this significant increase in airway smooth muscle was noted in both small airways (OVA/OVA, $7.05 \pm 0.76 \mu$ m²/ μ m versus OVA/PBS, $4.89 \pm 0.23 \mu$ m²/ μ m; $P < 0.05$) and intermediate-sized airways (OVA/OVA, $7.67 \pm 0.56 \mu$ m²/ μ m versus OVA/PBS, $5.27 \pm 0.48 \mu$ m²/ μ m; $P < 0.05$; Figure 2D, *right*). Although a trend was observed toward increased smooth muscle in the large airways as well, a statistical analysis could not be undertaken because of insufficient sample size. In contrast, in the acute model (Figures 2C and 2D, *left*), no significant difference was noted in the bronchial smooth muscle area of OVA-sensitized and OVA-challenged mice versus PBS-challenged mice.

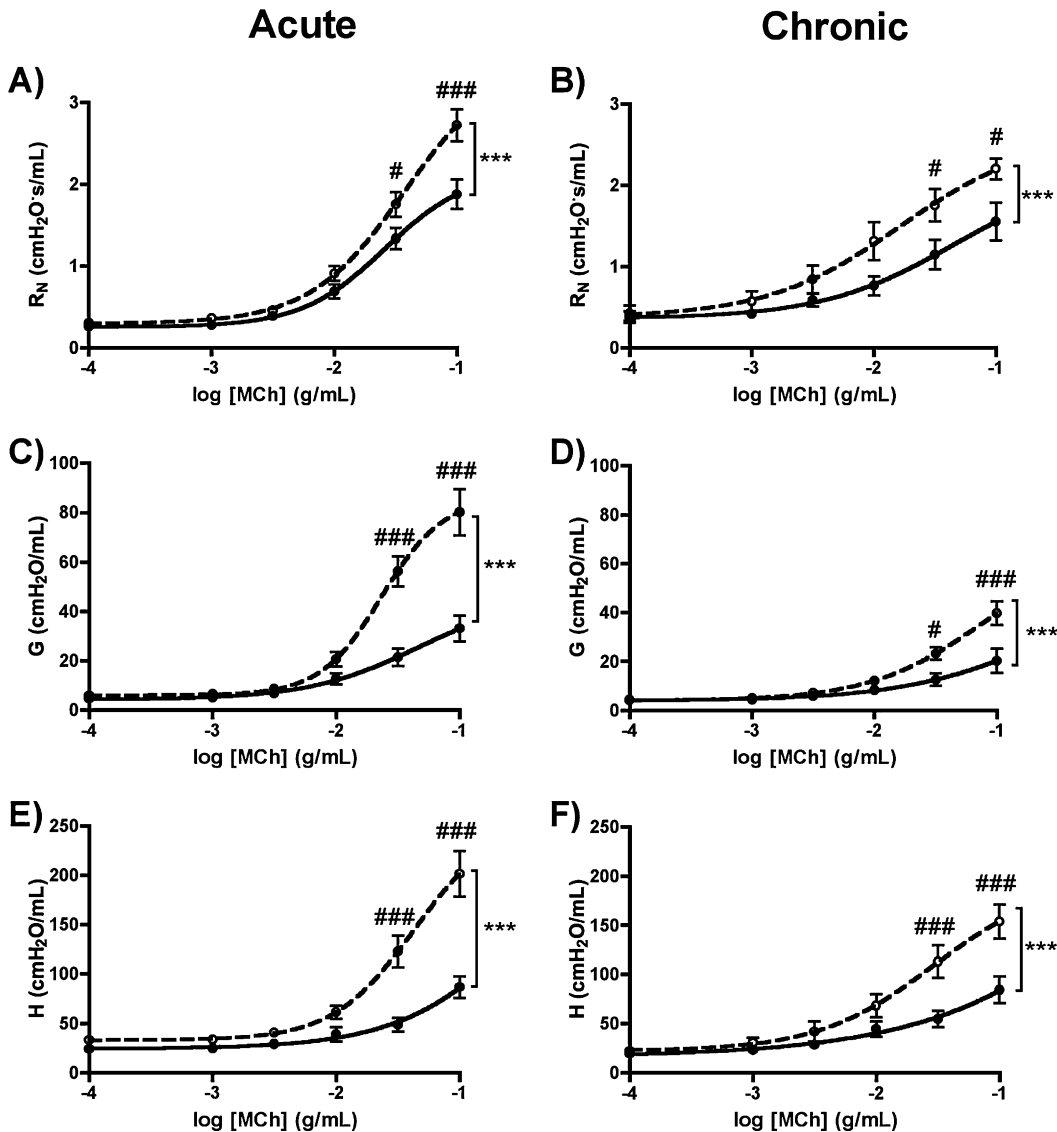


Figure 1. Airway hyperresponsiveness to methacholine in acute (3-week) and chronic (12-week) models of allergic airways inflammation. The airway (Newtonian) resistance (R_N ; in A and B) was significantly augmented in the ovalbumin (OVA)-sensitized and OVA-challenged (OVA/OVA; dashed lines) mice in both the acute and chronic models, compared with the respective OVA-sensitized and PBS-challenged control mice (OVA/PBS; solid lines). Measures of peripheral tissue damping (G; in C and D) and elastance (H; in E and F) were also significantly increased in the OVA/OVA mice (dashed lines) in the acute and chronic models, compared with OVA/PBS mice (solid lines). # $p < 0.05$ and ### $p < 0.001$, OVA/OVA versus OVA/PBS, assessed by two-way ANOVA. *** $p < 0.001$ between OVA/OVA and OVA/PBS methacholine dose-response curves according to F-test. Data are expressed as means $\pm$ SEM. $n = 11$ and 12 mice in the acute OVA/PBS and OVA/OVA treatment cohorts, respectively. $n = 16$ mice in each of the chronic OVA/OVA and OVA/PBS treatment cohorts (MCh, methacholine).

Airway Smooth Muscle Content Correlates with Airway Newtonian Resistance in the Chronic (12-Week) Model of Ovalbumin-Induced Allergic Asthma

To determine whether the functional endpoints were related to airway smooth muscle mass, we calculated the Spearman correlations between the responsiveness to methacholine (submaximal response to 30 mg/ml methacholine and maximal R_N , G, and H) and the small, intermediate, and all-airways morphometric data. Although none of the parameters indicating responsiveness to methacholine correlated with any of the smooth muscle content in the acute model, in the chronic model, the airway R_N to 100 mg/ml nebulized methacholine correlated directly with the smooth muscle content of all airways ($\rho = 0.77$, $P < 0.05$; Figure 3).

Hypertrophy Contributes to Increased Peripheral Airway Smooth Muscle Mass in the Chronic (12-Week) Model of Ovalbumin-Induced Allergic Asthma, whereas Increased Proliferation and Diminished Apoptosis Are Not Evident

To determine the relative contributions of ASMC proliferation and hypertrophy to the increased smooth muscle area, we immunostained lung sections from acute and chronic OVA/OVA and

OVA/PBS mice with α -smooth muscle actin (to measure total area), 4'-6-diamidino-2-phenylindole (DAPI) (for total nuclear number), and Ki67 (for proliferating nuclei) (Figure 4A).

As an index of hypertrophy, the total smooth muscle area was quantified by morphometry, and the number of smooth muscle cells (i.e., DAPI-positive nuclei within the smooth muscle) was counted. Smooth muscle area was divided by total nuclear number to determine muscle area/single cell, and then normalized to airway basement membrane length. When all airways were considered together, a significant increase was evident in smooth muscle cell area in the OVA/OVA mice compared with OVA/PBS mice in the chronic 12-week model ($0.128 \pm 0.013 \mu\text{m}^2/\mu\text{m}$ versus $0.086 \pm 0.012 \mu\text{m}^2/\mu\text{m}$, respectively; $P < 0.05$; Figure 4B, right), which was not observed in the acute model (Figure 4B, left). When myocyte size was considered in the predefined airway size cohorts to assess for regional variations across the bronchial tree, we observed an increase in smooth muscle cell area in both the small airways (OVA/OVA, $0.130 \pm 0.013 \mu\text{m}^2/\mu\text{m}$ versus OVA/PBS, $0.087 \pm 0.015 \mu\text{m}^2/\mu\text{m}$; $P < 0.05$; Figure 4C, right) and intermediate-sized airways (OVA/OVA, $0.113 \pm 0.009 \mu\text{m}^2/\mu\text{m}$ versus OVA/PBS, $0.078 \pm 0.013 \mu\text{m}^2/\mu\text{m}$; $P < 0.05$) in the chronic model. A maximum of one large airway fulfilled the inclusion criteria for morphometric assessment per mouse.

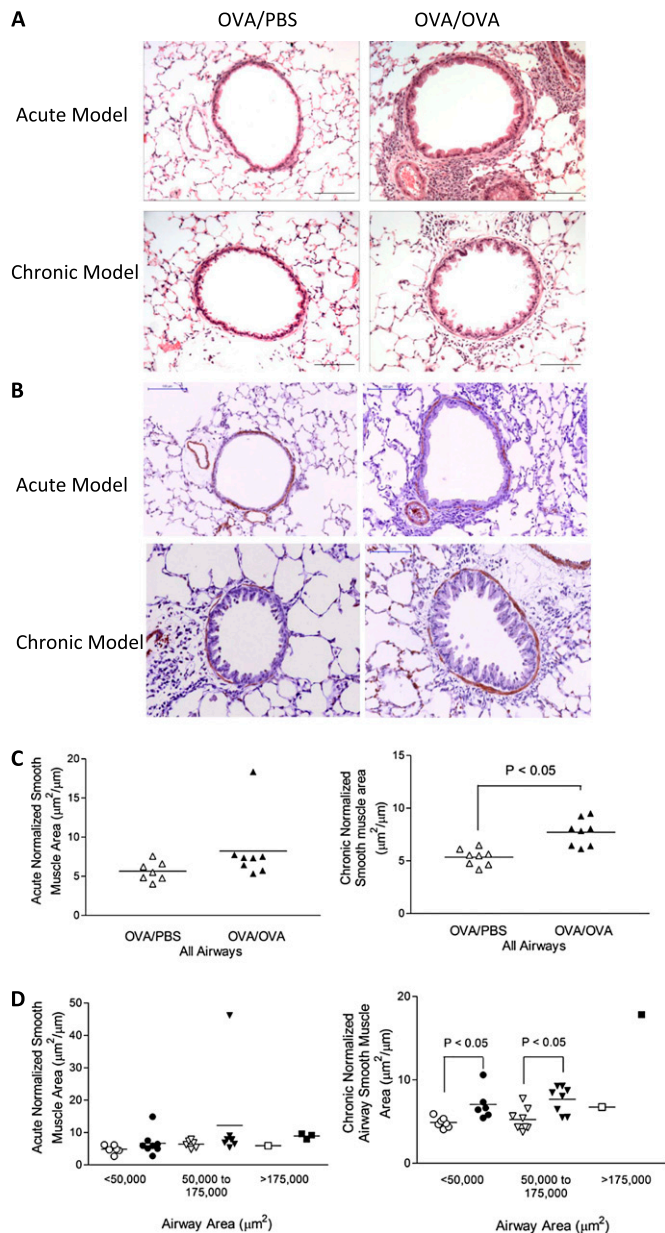


Figure 2. α -smooth muscle actin is increased in the airways of the chronic murine model of allergic asthma. (A) Formalin-fixed, hematoxylin and eosin-stained histologic sections of lungs from mice sensitized with OVA and serially challenged with saline (OVA/PBS) or OVA (OVA/OVA) for 3 weeks (acute model) or 12 weeks (chronic model) reveal a robust inflammatory reaction in the acute OVA/OVA mice that resolves in the chronic OVA/OVA mice. (B) Serial sections were immunostained for α -smooth muscle actin (brown staining). (C) The airway α -smooth muscle actin area was quantified and then normalized to basement membrane length. A significant increase was evident in the α -smooth muscle actin area of the OVA/OVA mice (solid symbols) versus OVA/PBS mice (open symbols) in the chronic model, when all airways were considered together (right; $P < 0.05$). (D) This difference was seen throughout the bronchial tree from small (area, $< 50,000 \mu\text{m}^2$; right; $P < 0.05$) to intermediate-sized (area, $50,000$ – $175,000 \mu\text{m}^2$; $P < 0.05$) airways. In contrast, no significant difference was evident in α -smooth muscle actin area in the airways of acute OVA/OVA mice (solid symbols) versus OVA/PBS mice (open symbols) (C and D, left). Scale bar = $100 \mu\text{m}$ ($n = 7$ mice in the acute OVA/PBS cohort; $n = 8$ mice per group for all other cohorts).

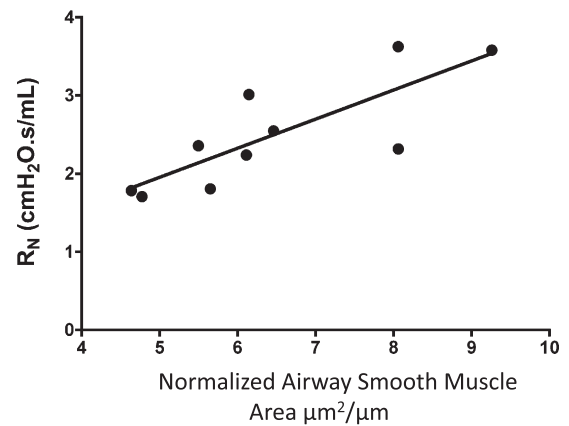


Figure 3. Airway smooth muscle content correlates with airway Newtonian resistance in the chronic (12-week) murine model of ovalbumin-induced allergic asthma. Scatterplot indicates the methacholine-induced changes in airway Newtonian resistance (R_N) versus normalized airway smooth muscle area (ASM) in all airways in the chronic 12-week model. ASM area correlates with R_N in all airways ($P < 0.05$) in the OVA/OVA-treated mice ($n = 10$ mice).

Thus a representative sample was not available for each animal, and this cohort was excluded from the analysis. No differences were observed across the various airway size cohorts in the acute model (Figure 4C, left).

To quantify proliferation in the airway smooth muscle, Ki67-positive (Ki67⁺) nuclei were counted, expressed as a ratio of the total number of DAPI-positive nuclei, and normalized to airway basement membrane length. In the acute 3-week model, a significant increase was evident in smooth muscle cell proliferation across all airways in the OVA-sensitized and OVA-challenged mice ($1.70 \times 10^{-4} \pm 0.60 \times 10^{-4}$ Ki67⁺/μm) versus saline-challenged mice ($0.15 \times 10^{-4} \pm 0.09 \times 10^{-4}$ Ki67⁺/μm; $P < 0.05$; Figure 4D, left). Analysis within the predetermined airway size cohorts to assess for regional variation across the bronchial tree revealed an increase in proliferation in both small airways (OVA/OVA, $1.96 \times 10^{-4} \pm 0.66 \times 10^{-4}$ Ki67⁺/μm versus OVA/PBS, $0.19 \times 10^{-4} \pm 0.11 \times 10^{-4}$ Ki67⁺/μm; $P < 0.05$) and intermediate-sized airways (OVA/OVA, $1.06 \times 10^{-4} \pm 0.39 \times 10^{-4}$ Ki67⁺/μm versus OVA/PBS, $0.050 \times 10^{-4} \pm 0.02 \times 10^{-4}$ Ki67⁺/μm; $P < 0.05$; Figure 4E, left). In contrast, no significant muscle proliferation was noted in the airways of mice in the chronic 12-week model (Figures 4D and 4E, right).

Apoptosis plays an important role in controlling cell numbers in various tissues and organs. For example, both the inhibition of apoptosis and increased pulmonary artery smooth muscle cell proliferation contribute to increases in pulmonary vascular smooth muscle mass (27). To quantify apoptosis in our acute and chronic models of murine allergic asthma, TUNEL-positive nuclei (Figure 5A) in airway smooth muscle were counted, expressed as a ratio of the total number of nuclei, and normalized to basement membrane length. The increase in smooth muscle area in chronic OVA/OVA-treated mice was likely not a consequence of diminished cell death attributable to apoptosis, because the ratio of TUNEL-positive nuclei was not significantly different between chronic OVA/OVA and OVA/PBS mice (Figures 5B and 5C, right). In contrast, a significant decrease was evident in the number of TUNEL-positive (TUNEL⁺) nuclei in the acute OVA/OVA mice versus OVA/PBS mice ($0.13 \times 10^{-4} \pm 0.10 \times 10^{-4}$ TUNEL⁺/μm versus $0.81 \times 10^{-4} \pm 0.15 \times 10^{-4}$ TUNEL⁺/μm, respectively; $P < 0.05$; Figure 5B, left) when all airways were considered together.

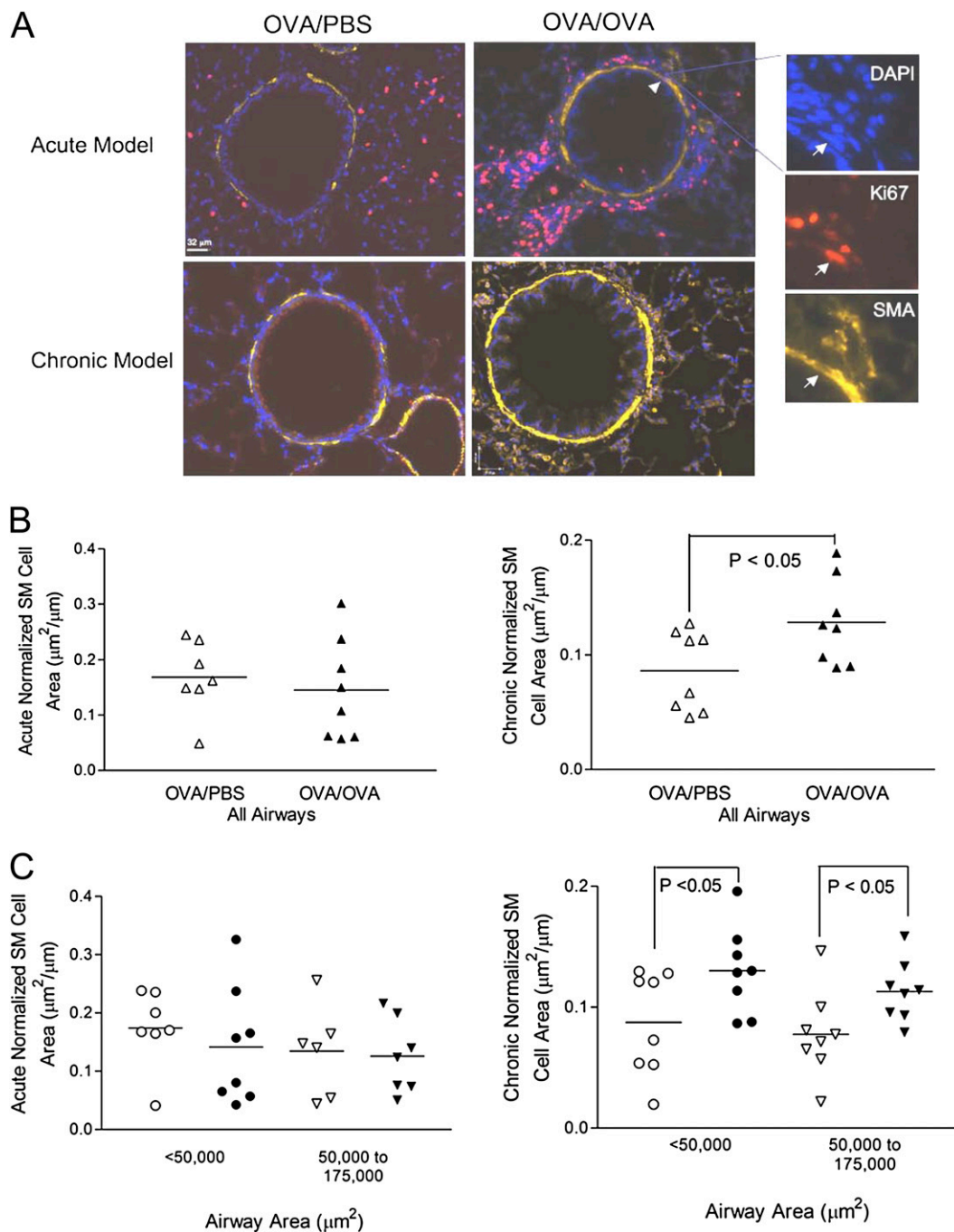


Figure 4. Assessment of cellular proliferation and hypertrophy in the airway smooth muscle of acute and chronic murine models of OVA-induced asthma. (A) Formalin-fixed histologic sections of lungs from acute and chronic OVA/PBS and OVA/OVA mice were immunostained for α -smooth muscle actin (yellow), 4'-6-diamidino-2-phenylindole (blue) for total nuclei, and Ki67 (red) for proliferating nuclei. The arrowhead indicates the portion of airway wall magnified to demonstrate a representative Ki67-positive nucleus (arrows) within smooth muscle. (B and C) The smooth muscle area (yellow) was quantitated through morphometry and divided by the total number of nuclei within the smooth muscle, to determine the average smooth muscle cell area, and then normalized to airway basement membrane length. A significant increase was evident in the smooth muscle area per cell in all airways in chronic OVA/OVA mice (solid symbols) versus OVA/PBS mice (open symbols; B, right). When airways were categorized based on size in the chronic model, the increase in smooth muscle area/cell of the OVA/OVA mice (solid symbols) versus OVA/PBS mice (open symbols) was evident throughout the bronchial tree in small (area, < 50,000 μm^2) and intermediate-sized (area, 50,000–175,000 μm^2) airways (C, right). In contrast, no difference was detected in the acute-model airways between OVA/OVA mice (solid symbols) and OVA/PBS mice (open symbols) (B and C, left). (D and E) The numbers of Ki67-positive (Ki67+ve) nuclei

in airway smooth muscle were counted, expressed as a ratio of total nuclei, and normalized to basement membrane length. A significant increase was evident in the number of proliferating nuclei across all airways in OVA/OVA mice (solid symbols) versus OVA/PBS mice (open symbols) in the acute model (D, left). This increase was consistent throughout the bronchial tree in the small and intermediate-sized airways (E, left). No significant difference was evident in the number of proliferating nuclei in airway smooth muscle of mice in the chronic model (C and D, right). Approximately 10 fields of view ($\times 40$ magnification) were counted for each section, representing > 500 nuclei. SMA, smooth muscle actin. Scale bar = 100 μm ($n = 7$ mice in the acute OVA/PBS cohort; $n = 8$ mice per group in all other cohorts).

Further analysis revealed that this decrease occurred throughout the bronchial tree in the small and intermediate-sized airways in the acute OVA/OVA mice ($0.14 \times 10^{-4} \pm 0.12 \times 10^{-4}$ TUNEL $^{+}/\mu\text{m}$ and $0.05 \times 10^{-4} \pm 0.04 \times 10^{-4}$ TUNEL $^{+}/\mu\text{m}$, respectively; Figure 5C, left) versus OVA/PBS mice ($0.77 \times 10^{-4} \pm 0.20 \times 10^{-4}$ TUNEL $^{+}/\mu\text{m}$ and $0.64 \times 10^{-4} \pm 0.12 \times 10^{-4}$ TUNEL $^{+}/\mu\text{m}$, respectively; $P < 0.05$). A maximum of one large airway fulfilled the inclusion criteria for morphometric assessment per mouse. Thus a representative sample was not

available for each animal, and this cohort was excluded from the analysis.

Smooth Muscle Proliferative Index Correlates with Peripheral Tissue Damping and Elastance in the 3-Week Acute Model of Ovalbumin-Induced Allergic Asthma

To determine whether the functional endpoints were related to airway smooth muscle proliferation or apoptosis, we calculated

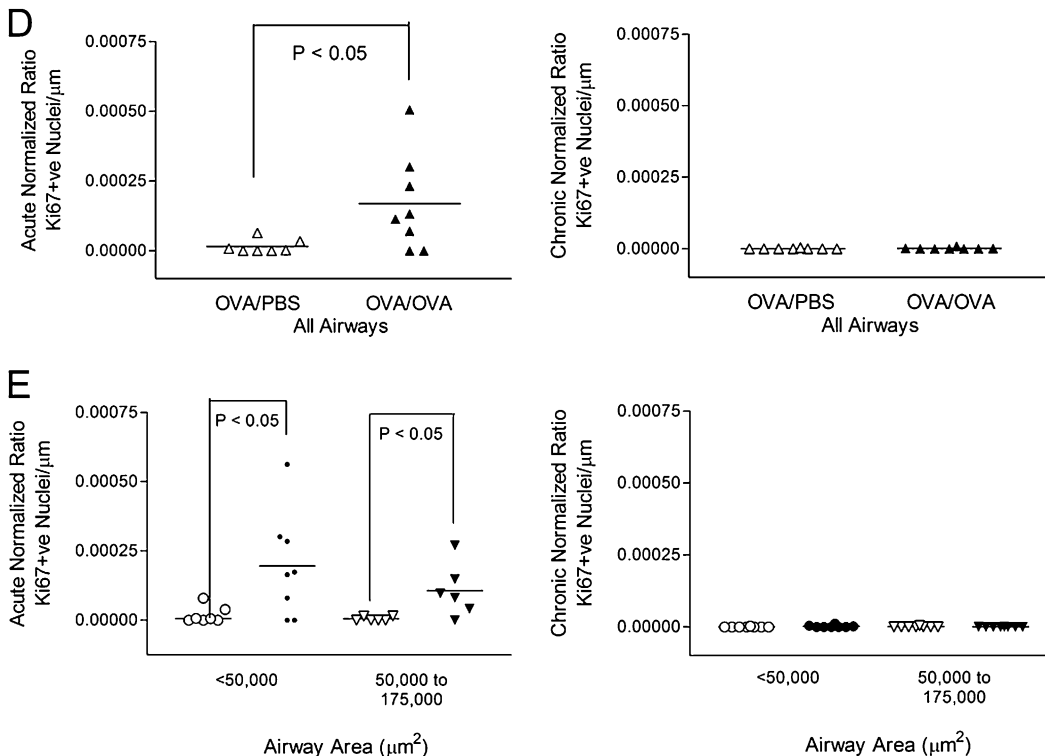


Figure 4. (Continued).

the Spearman correlations between the responsiveness to methacholine (R_N , G, and H) and the small, intermediate, and all-airways morphometric data. Although none of the parameters indicating responsiveness to methacholine correlated with the proliferative index in the chronic model, in the acute model, the maximum methacholine-induced increase in peripheral tissue elastance correlated directly with the proliferative index of smooth muscle in all airways ($\rho = 0.86$; $P < 0.05$) and small airways ($\rho = 0.86$; $P < 0.05$; Figure 6A). In addition, the maximum methacholine-induced increase in peripheral tissue damping correlated directly with the proliferative index of smooth muscle in all airways ($\rho = 0.96$; $P < 0.005$) and small airways ($\rho = 0.96$; $P < 0.005$; Figure 6B). None of the parameters indicating responsiveness to methacholine correlated with the TUNEL indices in the acute or chronic model.

DISCUSSION

This study investigated the relative contributions of smooth muscle hypertrophy, proliferation, and apoptosis to airway hyperresponsiveness in acute (3-week) and chronic (12-week) models of OVA-induced allergic airway inflammation/asthma in BALB/c mice. Using the flexiVent system and constant phase modeling described by Hantos and others (21–23), we were able to delineate changes in respiratory responsiveness, isolating contributions from the conducting central airways (airway R_N) versus peripheral lung tissue (G and H), thus providing a comprehensive investigation of lung mechanics beyond the determination of total respiratory system resistance, as classically employed in rodent studies of asthma (10, 22, 28, 29). Indices of increased airway hyperreactivity (AHR) were observed in both the acute and chronic models, consistent with previous reports (10–12, 20). Morphometric analyses demonstrated significant increases in airway smooth muscle area in the chronic model, which correlated directly with airway R_N in response to methacholine. In contrast, in the acute model, none of the parameters of

responsiveness to methacholine correlated with the airway smooth muscle content. Rather, the acute group exhibited active smooth muscle proliferation, which correlated with increased parameters of peripheral tissue damping and elastance. Concomitant diminished apoptosis was also observed. This response was mitigated in the chronic model, where the increased airway muscle mass demonstrated hypertrophic growth. These findings suggest that these two models represent the progression from an initial proliferative muscle response after acute allergen challenge to an increased muscle mass in which ASMC hypertrophy plays an important role in the airway remodeling response to chronic allergen challenge and bronchial hyperreactivity.

Previous studies demonstrated the temporality of the airway response in the murine allergic asthma model (11, 12, 26, 30, 31), with an early dominant inflammatory reaction that wanes over time, and the concomitant development of airway remodeling. Those studies did not, however, address the relative contributions of ASMC hypertrophy versus hyperplasia to the increased muscle mass. More recently, the presence of both hypertrophy and hyperplasia was reported in an OVA-sensitized and OVA-challenged murine model assessing the smooth muscle phenotype at a point (4 weeks) intermediate between our acute (3-week) and chronic (12-week) models (28). When considered together with the present study, these data support the premise that the initial muscle response in the murine model of allergic asthma is proliferative, antiapoptotic, and self-limited, whereas airway smooth muscle hypertrophy is a later, temporally distinct phenomenon.

Although we demonstrated in the chronic model that the increased airway smooth muscle content correlates highly with AHR centered specifically upon the airways (R_N), interestingly, the only positive correlations between muscle assessments and functional outcomes in the acute model were between the proliferation index of airway smooth muscle and peripheral lung damping and elastance (i.e., measures of lung stiffness). We speculate that the increased proliferation index in airway smooth muscle is an indicator of the robust inflammatory, proliferative

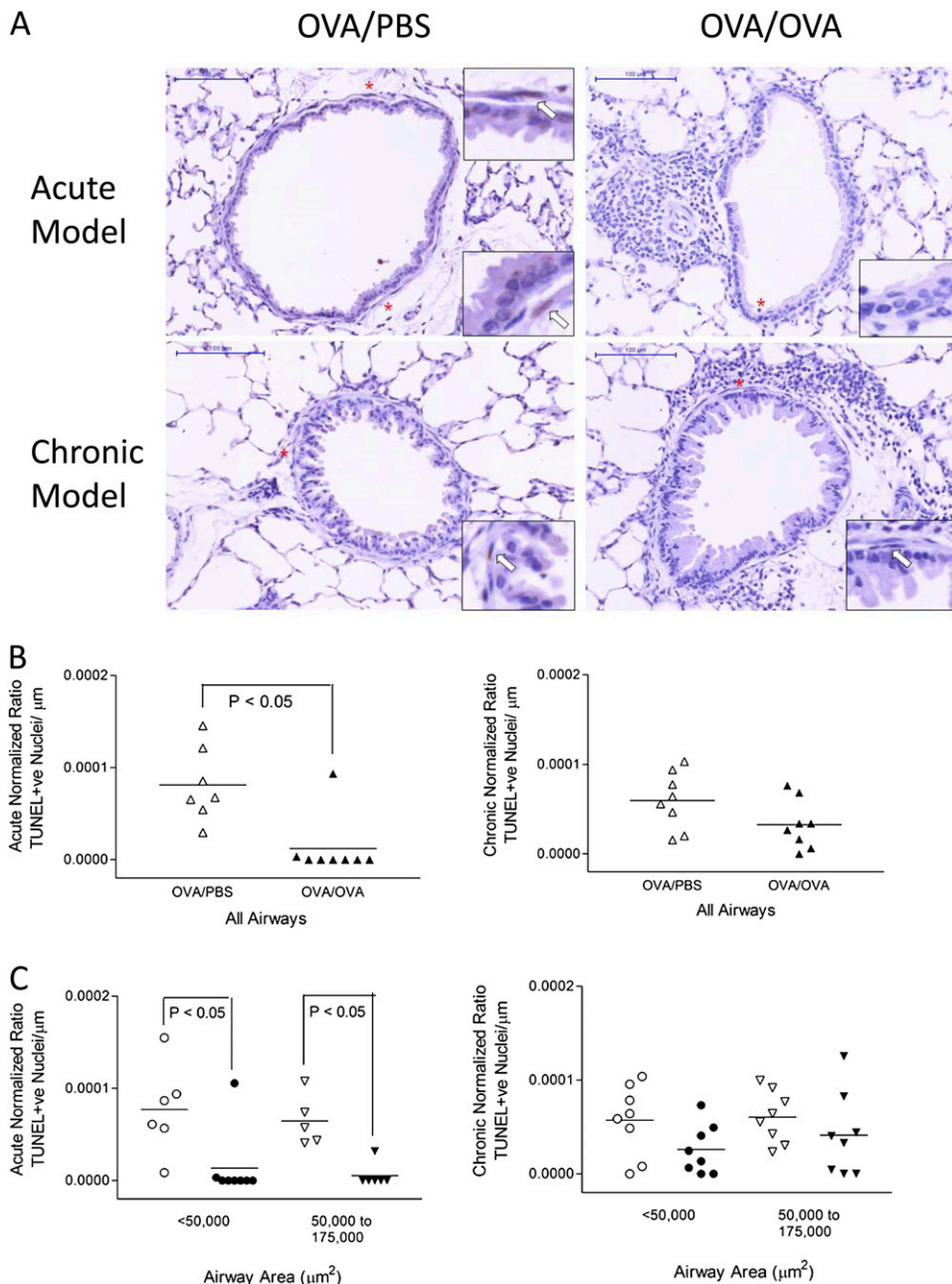


Figure 5. Apoptosis in the airway smooth muscle of acute and chronic murine models of OVA-induced asthma. (A) TdT-mediated dUTP nick-end label (TUNEL) staining of formalin-fixed histologic sections of lungs from acute and chronic OVA/PBS mice (open symbols) and OVA/OVA mice (solid symbols). Hematoxylin was used as a counterstain. TUNEL-positive apoptotic nuclei stain brown. Pink stars indicate the portion of airway wall magnified in the insets. Arrows indicate representative brown TUNEL-positive nuclei. (B) OVA challenge induced a significant decrease in the ratio of apoptotic nuclei in airway smooth muscle when all airways were considered together in acute OVA/OVA mice compared with acute OVA/PBS mice (B, left; $P < 0.05$). (C) There was no regional variation, because the decrease was evident in both small and intermediate-sized airways (C, left). Airways from OVA/OVA chronic model mice showed no significant change in apoptosis, compared with OVA/PBS mice (B and C, right). Approximately 10 fields of view ($\times 40$ magnification) were counted for each section, representing > 500 nuclei. Scale bar = $100 \mu\text{m}$ ($n = 7$ mice in the acute OVA/PBS cohort; $n = 8$ mice per group in all other cohorts).

response evident throughout the lung in the acute model. We further speculate that the increases in tissue damping and elastance demonstrate that AHR at this stage results, in part, from abnormalities in the peripheral lung tissue and airway inflammation and closure (24, 25). The lack of correlation between airway smooth muscle measures and R_N suggests that the increased airway resistance in the acute model does not result from abnormalities of the airway smooth muscle alone. Others assessed the relative contributions of inflammation and airway remodeling to AHR in a murine model of allergic asthma (11, 12, 30), and reported that AHR is associated with inflammation and immune-mediated events in the acute stages, but in the long term, the AHR results from airway remodeling. The vast majority of these studies, however, assessed total respiratory system resistance as opposed to separating out airway and tissue mechanics. To date, we are aware of only four studies that used the constant phase model to investigate the relative contributions of inflammation and airway remodeling to the alterations in AHR seen in the

murine model of allergic asthma (21, 24, 25, 31). These studies variably demonstrated an acute inflammatory response throughout the lung, with airway mucosal thickening attributable to edema, epithelial hyperplasia (24), and extracellular matrix (31) deposition associated with increases in H and G (21, 24, 25, 31) and, in some instances, R_N (24, 25, 31). The enhanced AHR in the acute time frame appears to result from these inflammatory changes and the propensity to airway closure, as opposed to changes in smooth muscle content or intrinsic contractility (21, 24, 25, 31). Airway smooth muscle remodeling with increased mass is not evident in the acute time frame in any of these studies, and is evident only in chronic models.

The relevance of our present findings to human disease becomes apparent when considering that, as with the murine model of allergen-induced asthma, the airways in human asthma exhibit both smooth muscle hypertrophy and hyperplasia (6, 7, 19). ASMCs isolated from patients with asthma and cultured *in vitro* retain an enhanced capacity for hypertrophic growth

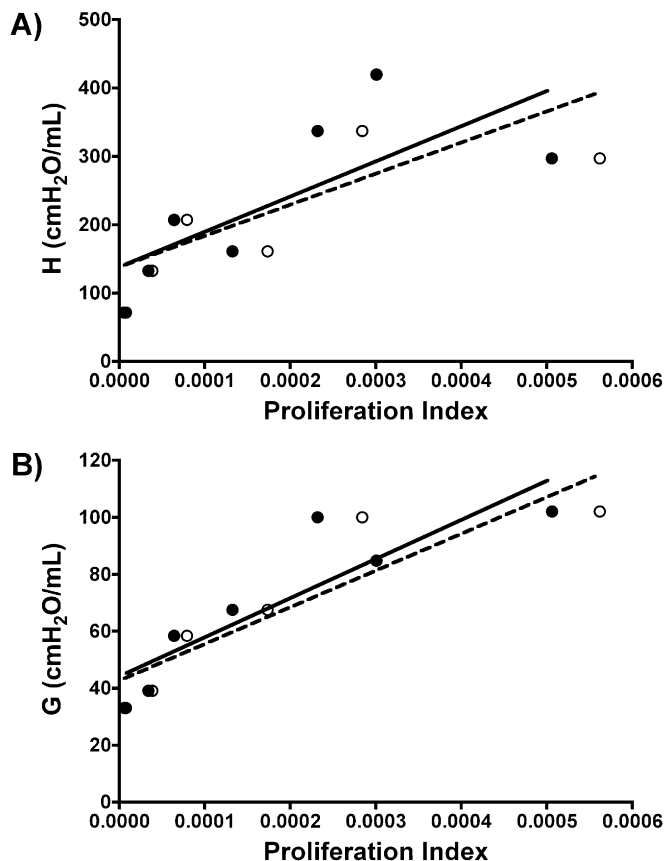


Figure 6. Airway smooth muscle proliferation index correlates with peripheral tissue damping (G) and elastance (H) in the acute (3-week) murine model of OVA-induced allergic asthma. Scatterplots indicate the methacholine-induced changes in (A) peripheral tissue elastance and (B) peripheral tissue damping versus the airway proliferation index in all (solid symbols and solid lines) and small (open symbols and dashed lines) airways, respectively, in the acute 3-week model. The maximum methacholine-induced increase in peripheral tissue elastance correlated directly with the proliferative index of smooth muscle in all airways, and specifically the small airways ($P < 0.05$) of OVA/OVA-treated mice. The maximum methacholine-induced increase in peripheral tissue damping correlated directly with the proliferative index of smooth muscle in all airways and, specifically, in the small airways ($P < 0.005$) of OVA/OVA-treated mice ($n = 7$ mice).

and an enhanced proliferative capacity (16, 18, 32–34). These “asthmatic” ASMCs were also reported to be hypercontractile *in vitro* and thus primed to respond to bronchoconstricting stimuli (17). However, in contrast to the murine models, the relative contributions of hypertrophic versus hyperplastic muscle to airflow obstruction in human asthma remain controversial. For example, some studies assessing endobronchial biopsies from patients with differing severities of asthma reported that airway smooth muscle hypertrophy and fibroblast accumulation were selective determinants of severe, persistent asthma (6), whereas others noted that both ASMC hyperplasia and hypertrophy comprise the increased airway smooth muscle mass in severe disease (8). In addition, whether a temporal sequence is involved with the development of hyperplasia and hypertrophy in humans remains unknown. Similar to the murine model of chronic disease, human studies have demonstrated that airway lability occurs in association with airway remodeling (including smooth muscle hypertrophy) in the absence of evidence of ongoing airway inflammation (8). Although this result may be partly attributable to an individual’s use of long-term oral and high-dose inhaled glucocorticoid

therapy, these findings raise the possibility that remodeling occurs independent of inflammation.

Determination of the airway-muscle processes that influence AHR and airflow limitation suggest that current approaches to the treatment of asthma may require reassessment. Attenuation of the inflammatory response and stimulation of bronchodilation remain the mainstays of asthma therapy, but no treatment, short of laser ablation of airway muscle (35, 36), is currently aimed specifically at diminishing the increased muscle mass, or inducing atrophy, of ASMCs. Signaling networks that regulate pulmonary smooth muscle hypertrophy are currently being delineated, and the activation of the well-characterized AKT/glycogen synthase kinase (GSK) β pathway, known to induce a hypertrophic response in skeletal muscle (37, 38), was recently recognized to induce hypertrophy of airway smooth muscle as well (13, 28). The increased proliferative rate described in human ASMCs was suggested to result from dysfunctional translational regulation of the CCAAT/enhancer binding protein (C/EBP- α) (18, 33), in addition to Ca^{2+} -induced enhanced mitochondrial biogenesis (32). Developing pharmacologic therapies to influence the specific cellular signaling pathways regulating airway smooth muscle hypertrophy and proliferation is critical, would complement anti-inflammatory and bronchodilator therapies, and likely would favorably influence outcomes in those with moderate to severe persistent asthma.

This study contains some limitations. Although we demonstrated a hypertrophic smooth muscle mass in the airways of mice that correlates with airway hyperreactivity in allergic asthma, we did not directly demonstrate the hypercontractility of these muscle cells using *ex vivo* or *in vitro* methods. Other investigators assessed the contractile properties of hypertrophic bronchial smooth muscle cells *in vitro*, and found that increases in contractile protein content result in increased smooth muscle cell contractile shortening in response to acetylcholine and KCl (13, 14), supporting our premise. Furthermore, our OVA dosing regimen was not identical between our two models, with 7 consecutive days of OVA dosing administered in the acute model, and two consecutive daily doses administered every 2 weeks for 3 months in the chronic model. It is unlikely that the absence of proliferation in the chronic model simply reflects this different dosing regimen, because other short-term studies reported that only 3 consecutive days of OVA treatment can induce proliferation (28). We speculate that the loss of stimulation to a proliferative response may result from the induction of tolerance to the allergen during chronic, repeated exposures. Finally, although the constant phase model provides a means by which to distinguish between the resistance provided by the conducting airways versus measures of peripheral lung stiffness (G and H), this model, as with systems measuring total respiratory resistance, does not rule out the influence of the thoracic cage and chest wall on pulmonary mechanics, because we did not assess intrapleural pressures.

Overall, we have shown that the proliferative, apoptotic, and hypertrophic responses in airway smooth muscle are temporally distinct in a murine model of allergic asthma. Proliferation with diminished apoptosis comes first as part of a large inflammatory reaction to allergen challenge, followed by the resolution of proliferation, the normalization of apoptosis, and the increased synthesis of contractile proteins, with hypertrophy of pulmonary smooth muscle. This increased, hypertrophied muscle mass correlated strongly with an index of methacholine hyperreactivity that is centered on the airway tree (Newtonian resistance). The identification of a hypertrophic muscle mass that is functionally important indicates that ongoing investigation should focus on the regulation of pulmonary smooth muscle hypertrophy as a component of asthma management.

Author disclosures are available with the text of this article at www.atsjournals.org.

References

- Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM. Asthma: from bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med* 2000;161:1720–1745.
- Busse W, Elias J, Sheppard D, Banks-Schlegel S. Airway remodeling and repair. *Am J Respir Crit Care Med* 1999;160:1035–1042.
- Elias JA, Zhu Z, Chupp G, Homer RJ. Airway remodeling in asthma. *J Clin Invest* 1999;104:1001–1006.
- Vignola AM, Kips J, Bousquet J. Tissue remodeling as a feature of persistent asthma. *J Allergy Clin Immunol* 2000;105:1041–1053.
- Vignola AM, Chané P, Bonsignore G, Godard P, Bousquet J. Structural consequences of airway inflammation in asthma. *J Allergy Clin Immunol* 2000;105:S514–S517.
- Benayoun L, Druihe A, Dombret MC, Aubier M, Pretolani M. Airway structural alterations selectively associated with severe asthma. *Am J Respir Crit Care Med* 2003;167:1360–1368.
- Woodruff PG, Dolganov GM, Ferrando RE, Donnelly S, Hays SR, Solberg OD, Carter R, Wong HH, Cadbury PS, Fahy JV. Hyperplasia of smooth muscle in mild to moderate asthma without changes in cell size or gene expression. *Am J Respir Crit Care Med* 2004;169:1001–1006.
- Jenkins HA, Cool C, Szeffler SJ, Covar R, Brugman S, Gelfand EW, Spahn JD. Histopathology of severe childhood asthma: a case series. *Chest* 2003;124:32–41.
- Lambert RK, Wiggs BR, Kuwano K, Hogg JC, Pare PD. Functional significance of increased airway smooth muscle in asthma and COPD. *J Appl Physiol* 1993;74:2771–2781.
- Henderson WR Jr, Tang LO, Chu SJ, Tsao SM, Chiang GK, Jones F, Jonas M, Pae C, Wang H, Chi EY. A role for cysteinyl leukotrienes in airway remodeling in a mouse asthma model. *Am J Respir Crit Care Med* 2002;165:108–116.
- Southam DS, Ellis R, Wattie J, Inman MD. Components of airway hyperresponsiveness and their associations with inflammation and remodeling in mice. *J Allergy Clin Immunol* 2007;119:848–854.
- Leigh R, Ellis R, Wattie J, Southam DS, De HM, Gaudie J, O'Byrne PM, Inman MD. Dysfunction and remodeling of the mouse airway persist after resolution of acute allergen-induced airway inflammation. *Am J Respir Cell Mol Biol* 2002;27:526–535.
- Deng H, Dokshin GA, Lei J, Goldsmith AM, Bitar KN, Fingar DC, Hershenson MB, Bentley JK. Inhibition of glycogen synthase kinase-3 β is sufficient for airway smooth muscle hypertrophy. *J Biol Chem* 2008;283:10198–10207.
- Goldsmith AM, Bentley JK, Zhou L, Jia Y, Bitar KN, Fingar DC, Hershenson MB. Transforming growth factor- β induces airway smooth muscle hypertrophy. *Am J Respir Cell Mol Biol* 2006;34:247–254.
- Halayko AJ, Solway J. Molecular mechanisms of phenotypic plasticity in smooth muscle cells. *J Appl Physiol* 2001;90:358–368.
- Johnson PR, Roth M, Tamm M, Hughes M, Ge Q, King G, Burgess JK, Black JL. Airway smooth muscle cell proliferation is increased in asthma. *Am J Respir Crit Care Med* 2001;164:474–477.
- Ma X, Cheng Z, Kong H, Wang Y, Unruh H, Stephens NL, Laviolette M. Changes in biophysical and biochemical properties of single bronchial smooth muscle cells from asthmatic subjects. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L1181–L1189.
- Borger P, Miglino N, Baraket M, Black JL, Tamm M, Roth M. Impaired translation of CCAAT/enhancer binding protein α mRNA in bronchial smooth muscle cells of asthmatic patients. *J Allergy Clin Immunol* 2009;123:639–645.
- Ebina M, Takahashi T, Chiba T, Motomiya M. Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma: a 3-D morphometric study. *Am Rev Respir Dis* 1993;148:720–726.
- North ML, Khanna N, Marsden PA, Grasemann H, Scott JA. Functionally important role for arginase 1 in the airway hyperresponsiveness of asthma. *Am J Physiol Lung Cell Mol Physiol* 2009;296:L911–L920.
- Tomioka S, Bates JH, Irvin CG. Airway and tissue mechanics in a murine model of asthma: alveolar capsule vs. forced oscillations. *J Appl Physiol* 2002;93:263–270.
- Bates JH, Rincon M, Irvin CG. Animal models of asthma. *Am J Physiol Lung Cell Mol Physiol* 2009;297:L401–L410.
- Hantos Z, Daroczy B, Suki B, Nagy S, Fredberg JJ. Input impedance and peripheral inhomogeneity of dog lungs. *J Appl Physiol* 1992;72:168–178.
- Wagers S, Lundblad LK, Ekman M, Irvin CG, Bates JH. The allergic mouse model of asthma: normal smooth muscle in an abnormal lung? *J Appl Physiol* 2004;96:2019–2027.
- Lundblad LK, Thompson-Figueroa J, Allen GB, Rinaldi L, Norton RJ, Irvin CG, Bates JH. Airway hyperresponsiveness in allergically inflamed mice: the role of airway closure. *Am J Respir Crit Care Med* 2007;175:768–774.
- Hirota JA, Ellis R, Inman MD. Regional differences in the pattern of airway remodeling following chronic allergen exposure in mice. *Respir Res* 2006;7:120.
- Cowan KN, Jones PL, Rabinovitch M. Regression of hypertrophied rat pulmonary arteries in organ culture is associated with suppression of proteolytic activity, inhibition of tenascin-C, and smooth muscle cell apoptosis. *Circ Res* 1999;84:1223–1233.
- Bentley JK, Deng H, Linn MJ, Lei J, Dokshin GA, Fingar DC, Bitar KN, Henderson WR Jr, Hershenson MB. Airway smooth muscle hyperplasia and hypertrophy correlate with glycogen synthase kinase-3 β phosphorylation in a mouse model of asthma. *Am J Physiol Lung Cell Mol Physiol* 2009;296:L176–L184.
- Bentley JK, Hershenson MB. Airway smooth muscle growth in asthma: proliferation, hypertrophy, and migration. *Proc Am Thorac Soc* 2008;5:89–96.
- Locke NR, Royce SG, Wainwright JS, Samuel CS, Tang ML. Comparison of airway remodeling in acute, subacute, and chronic models of allergic airways disease. *Am J Respir Cell Mol Biol* 2007;36:625–632.
- Pinelli V, Marchica CL, Ludwig MS. Allergen-induced asthma in C57Bl/6 mice: hyper-responsiveness, inflammation and remodelling. *Respir Physiol Neurobiol* 2009;169:36–43.
- Triant T, Benard G, Begueret H, Rossignol R, Girodet PO, Ghosh D, Ousova O, Vernejoux JM, Marthan R, Tunon-de-Lara JM, et al. Bronchial smooth muscle remodeling involves calcium-dependent enhanced mitochondrial biogenesis in asthma. *J Exp Med* 2007;204:3173–3181.
- Roth M, Johnson PR, Borger P, Bihl MP, Rudiger JJ, King GG, Ge Q, Hostettler K, Burgess JK, Black JL, et al. Dysfunctional interaction of C/EBP α and the glucocorticoid receptor in asthmatic bronchial smooth-muscle cells. *N Engl J Med* 2004;351:560–574.
- Johnson PR, Burgess JK, Underwood PA, Au W, Poniris MH, Tamm M, Ge Q, Roth M, Black JL. Extracellular matrix proteins modulate asthmatic airway smooth muscle cell proliferation via an autocrine mechanism. *J Allergy Clin Immunol* 2004;113:690–696.
- Castro M, Rubin AS, Laviolette M, Fitterman J, De Andrade LM, Shah PL, Fiss E, Olivenstein R, Thomson NC, Niven RM, et al. Effectiveness and safety of bronchial thermoplasty in the treatment of severe asthma: a multicenter, randomized, double-blind, sham-controlled clinical trial. *Am J Respir Crit Care Med* 2010;181:116–124.
- Cox G, Thomson NC, Rubin AS, Niven RM, Corris PA, Siersted HC, Olivenstein R, Pavord ID, McCormack D, Chaudhuri R, et al. Asthma control during the year after bronchial thermoplasty. *N Engl J Med* 2007;356:1327–1337.
- Glass DJ. Skeletal muscle hypertrophy and atrophy signaling pathways. *Int J Biochem Cell Biol* 2005;37:1974–1984.
- Stitt TN, Drujan D, Clarke BA, Panaro F, Timofeyeva Y, Kline WO, Gonzalez M, Yancopoulos GD, Glass DJ. The IGF-1/P13K/AKT pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. *Mol Cell* 2004;14:395–403.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.