

Independent and combined effects of airway remodelling and allergy on airway responsiveness

Running title: Airway remodelling, allergy and bronchoconstriction

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ABSTRACT

Airway remodelling and allergic inflammation are key features of airway hyperresponsiveness in asthma, however their interrelationships are unclear. This study investigated the separate and combined effects of increased airway smooth muscle (ASM) layer thickness and allergy on airway hyperresponsiveness. We integrated a protocol of ovalbumin (OVA)-induced allergy into a non-inflammatory mouse model of ASM remodelling induced by conditional and airway-specific expression of TGF- α in Egr-1 deficient transgenic mice, which produced thickening of the ASM layer following ingestion of doxycycline. Mice were sensitised to OVA and assigned to one of four treatment groups: Allergy - normal chow diet and OVA challenge; Remodelling – doxycycline in chow and saline challenge; Allergy and Remodelling – doxycycline in chow and OVA challenge; and Control - normal chow diet and saline challenge. Airway responsiveness to methacholine and histology were assessed. Compared with the Control group, airway responsiveness to methacholine was increased in the Allergy group, independently of changes in wall structure, whereas airway responsiveness in the Remodelling group was increased independent of exposure to aeroallergen. The combined effects of allergy and remodelling on airway responsiveness were greater than either alone. There was a positive relationship between the thickness of the ASM layer with airway responsiveness, which was upward shifted in the presence of allergy. These findings support allergy and airway remodelling as independent causes of variable and excessive airway narrowing.

ABBREVIATIONS LIST

AHR, airway hyperresponsiveness; Alu-gel-S, Alum; ASM, airway smooth muscle; CCSP-rtTA, Clara cell secretory protein-reverse tetracycline-controlled transactivator; EGFR, epidermal growth factor receptor; Egr-1, early growth response-1; G, tissue damping; H, tissue elastance; i.p., intraperitoneal; IUGR, intrauterine growth restriction; LFOT, low-frequency forced oscillation technique; MCh, methacholine; OVA, ovalbumin; PAS, periodic acid-Schiff's reagent; P_{bm} , perimeter of the basement membrane; R_n , Newtonian resistance; tetO₇, tetracycline operator; TGF- α , transforming growth factor- α ; WA_i , inner airway wall; WA_o , outer airway wall; WA_t , total airway wall; Z_{rs} , respiratory impedance; ΔR_n , change in Newtonian resistance after methacholine.

PERSPECTIVE

- Disease trajectories in asthma are incompatible with the notion that allergic inflammation drives airway wall pathology, since airway smooth muscle (ASM) thickness is elevated early in life, remains relatively constant, and is not associated with duration of disease. We propose that the mechanism producing increased ASM thickness in asthma is independent of allergy and that separately determined airway remodelling and allergy is required to produce allergic asthma.
- This study used transgenic mouse models of ASM thickening and allergen exposure to demonstrate two independent contributors to increased airway responsiveness. The combined effects of remodelling and allergy increased airway responsiveness above that possible by either airway remodelling or allergen alone. Notably, the effect of allergy on increased airway responsiveness was seen at all levels of remodelling.

- 76 • Our findings support independent contributions of allergy and airway remodelling in
77 asthma that may be determined by separate genetic and/or environmental
78 susceptibilities.

INTRODUCTION

Asthma is associated with excessive airway narrowing (airway hyperresponsiveness, AHR) and is characterized pathologically by airway remodelling, particularly increased thickness of the airway smooth muscle (ASM) layer, and inflammation in the conducting airways. The increase in ASM thickness is related to the severity of asthma (1) and to the ability of airways to narrow *in vitro* (2). Airway inflammation in asthma is most often allergic in nature with a predominance of Th-2 lymphocytes and eosinophils (3), although non-eosinophilic forms also occur (4, 5).

A major risk factor for asthma in childhood is the early development of sensitivity to inhaled allergens (6). However not all atopic individuals develop asthma and the relationships between allergy, airway inflammation and increased ASM thickness are still not clear. Allergic (eosinophilic) airway inflammation and increased ASM thickness are seen together in large and small airways in individuals with asthma (7). Accordingly it has been hypothesized that allergic sensitization combined with an intrinsic abnormality of the epithelium (8) will result in chronic allergic airway inflammation and drive ASM remodelling via epithelial mesenchymal transition (9). The ASM may itself attract and activate inflammatory cells and mast cells, resulting in increased proliferation (10). An alternative hypothesis is that airway inflammation and increased ASM thickness are independent factors that together produce AHR in asthma (1), which fits well with observations that the ASM layer is thickened before diagnosis of asthma in childhood (11). To date, no study has been able to demonstrate the independent roles of ASM remodelling and allergic airway inflammation on AHR in the context of asthma.

The proposal that allergic inflammation is not necessarily the driver of increased ASM thickness and instead co-exists with remodelling, is strengthened by independent contributions from airway inflammation and ASM thickness in determining asthma severity based on patients response to therapy. Treatment of allergic airway inflammation with corticosteroids controls asthma symptoms and reduces AHR (12) while ablation of ASM by bronchial thermoplasty improves asthma control and quality of life and reduces the number of severe asthma exacerbations and the dose of oral corticosteroids (13). Although drugs that relax ASM relieve the symptoms of asthma, they do not prevent the recurrence of symptoms (12). Therefore, both airway inflammation and increased ASM thickness seem necessary for the development of AHR and asthma but the degree to which these pathologies are related is unclear since they are so closely associated within the airways of patients with asthma (7, 14). To investigate the independent contributions of allergy and increased ASM thickness on the development of AHR, we measured airway responsiveness in transgenic mice when the thickness of the ASM layer was increased independently of inflammation, in a model of allergy without airway remodelling, and finally in transgenic mice exposed to allergen to examine the combined effects of airway remodelling and allergy.

MATERIALS AND METHODS

Clara cell secretory protein-reverse tetracycline (doxycycline)-controlled transactivator (CCSP-rtTA)^{+/-}/(tetracycline operator (tetO))₇ transforming growth factor- α (TGF- α)^{+/-}/early growth response-1 (Egr-1)^{-/-} mice were generated on a BALB/c background as described previously (15). The CCSP-rtTA^{+/-} transgenic mouse line used drives expression of transgenes (TGF- α) in Clara cells of the conducting airways, as well as a subset of alveolar type II cells (16). Egr-1 deficiency augments ASM thickening to TGF- α , independent of inflammation, which is triggered by addition of doxycycline to the diet (17).

Mice were housed at the Telethon Kids Institute in specific pathogen-free environments and maintained on a 12:12 hour light:dark cycle and supplied with food and water *ad libitum*. This study was carried out in accordance with the recommendations of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition). The protocol was approved by the Telethon Kids Institute Animal Ethics Committee (Project Number 231).

Sensitization, diet and protocols

All mice were initially sensitized by 0.2 mL intraperitoneal (i.p.) injections containing 5 mg/mL of chicken egg ovalbumin (OVA; Sigma, St Louis, MO, USA) suspended in 50 mL of Alum (Alu-gel-S, Serva, Heidelberg, Germany) at 5 weeks of age (day 0) and at 7 weeks of age (day 14). After experimentation (see below), blood was collected via cardiac puncture for analysis of total IgE by ELISA as per the manufacturer's instructions (BD Biosciences, San Diego, CA, USA).

Concurrently with the sensitization protocol, at 6.5 weeks of age, mice were fed a diet of doxycycline in chow (600 mg/kg; Specialty Feeds, Glen Forrest, WA, Australia) or normal chow (Control) for 10 days until 8 weeks of age (Figure 1). Mice were subsequently randomised to receive a 1 % OVA or saline aerosol challenge for 30 min (day 21) (18). Animals therefore belonged to one of four treatment groups: Allergy (n = 11) - a diet of normal chow and exposure to a single OVA challenge; Remodelling (n = 10) - 10 day doxycycline diet and saline challenge; Allergy and Remodelling (n = 16) - doxycycline diet and OVA challenge; and Control (n = 10) - a diet of normal chow and saline challenge.

Plethysmography and forced oscillation technique

Twenty four hours after aerosol challenge (saline or OVA), mice were anaesthetised by an i.p. injection of ketamine and xylazine (0.4 and 0.02 mg/g of body weight respectively), tracheostomised (19) and mechanically ventilated at 400 breaths/min with a tidal volume of 10 mL/kg and 2 cmH₂O positive end-expiratory pressure in a plethysmographic chamber (19). During pauses in ventilation the trachea was occluded and the intercostal muscles electrically stimulated to induce inspiratory efforts. Thoracic gas volume (lung volume) from the four treatment groups was calculated from Boyle's law using tracheal and chamber pressure as previously described (19, 20).

Mice were then transferred to a flexiVent system (FX module 1, flexiWare version 7.5, SCIREQ, Montreal, QC, Canada) to assess responsiveness to methacholine (MCh, Acetyl- β -methacholine chloride, Sigma-Aldrich, St. Louis, MO). Mice were ventilated at 250 breaths/minute with a tidal volume of 8.8 mL/kg and a positive end expiratory pressure of 2 cmH₂O. Lung volume history was standardised via three slow inflation-deflation manoeuvres up to 20 cmH₂O transrespiratory pressure. Once stabilised on the ventilator, respiratory mechanics were measured using a modified low-frequency forced oscillation technique (LFOT) (21), that applies multiple frequencies simultaneously, ranging from 0.25 to 19.625 Hz. Respiratory impedance (Z_{rs}) was measured (20, 22) and the constant phase model (23) used to partition Z_{rs} into airway and parenchymal components; allowing calculation of Newtonian resistance (R_n), inertance, tissue damping (G) and elastance (H). In mice, R_n is equivalent to airway resistance because of the high compliance of the chest wall (19). The calibration procedure in the flexiVent FX software was used to correct for the resistance of the tracheal cannula. Inertance values were negligible after correction for the impedance of the cannula and were not reported.

The LFOT measurements were acquired once a min for 5 min to establish baseline respiratory mechanics, followed by 10 s aerosols of saline and increasing doses of MCh (maximum dose 30 mg/mL) delivered by the Aeroneb ultrasonic nebulizer (SCIREQ). After each challenge, LFOT measurements were similarly acquired once a min for 5 min with the peak response used for analyses. We calculated ΔR_n as the change in R_n after MCh (post MCh R_n – post saline R_n).

Airway morphometry and histology

After MCh challenge, mice were administered an i.p. injection of 0.2 mL of atropine (600 μ g/mL) and 10 min later were euthanized by an overdose of ketamine and xylazine. Lungs were fixed by intra-tracheal instillation of 4 % formaldehyde at a transrespiratory pressure of 10 cmH₂O. The left lobe was embedded in paraffin with the proximal end facing down. Sections from the central bronchus were stained using the Masson's trichrome technique to measure airway wall thickness (15), inflammation and fibrosis (15), and with periodic acid-Schiff's reagent (PAS) (24) to examine the presence of mucins in the surface epithelium. Analyses described below were performed separately by blinded observers.

The cross-sectional area of ASM layer, epithelium, lumen area, inner (WA_i), outer (WA_o) and total wall (WA_t), and internal perimeter of the basement membrane (P_{bm}) were measured using the newCast stereology software (version 4.2.7, Visiopharm A/S, Horsholm, Denmark). WA_i and WA_o were defined as the regions internal and external to the ASM respectively, while WA_t comprised the inner, outer and ASM compartments. The square roots of areas were then corrected by the P_{bm} , the standard index of airway size (25). Strong PAS-staining

positive pixels in the epithelial layer were enumerated and expressed as a percentage of the total pixels within the epithelial layer (24).

The number of inflammatory cells (neutrophils, eosinophils and lymphomononuclear cells) were assessed semi-quantitatively within the airway wall and parenchyma and scored: 0 (none); 1 (mild) and 2 (moderate). Finally, since doxycycline produces lung fibrosis in mice on doxycycline for more extended periods (3-6 weeks (17, 26)), fibrosis was also assessed semi-quantitatively as: 0 (none); 1 (mild); 2 (moderate) and 3 (severe).

Statistical analysis

Data were transformed where necessary to ensure that the assumptions of normality and homoscedasticity of variances for the parametric tests were satisfied. Where this was not possible equivalent non-parametric tests were used. For the MCh challenges at saline or maximal dose of MCh (30 mg/mL), analyses were conducted using two-way ANOVA with aerosol challenge (saline vs OVA) and treatment (Control vs doxycycline) as factors. For the structural measurements, analyses were conducted using two-way ANOVA with aerosol challenge (saline vs OVA) and treatment (Control vs doxycycline) as factors. Multiple linear regression was used to analyse the relationship between the thickness of the ASM layer and WA_i with ΔR_n , and the relationship between total serum IgE and ΔR_n . Semi-quantitative scores of inflammatory cells were analysed using the chi-squared test. All data are presented as mean $\pm$ SEM with $*P < 0.05$ considered significant. Graphical and statistical analyses were performed using SigmaPlot version 13.0 and GraphPad Prism version 7.02. n refers to the number of mice.

RESULTS

Group characteristics: Details regarding sex, weight and lung volume are provided in Table 1. There was no difference in the sex ratio between groups or any independent effect of doxycycline (i.e., Remodelling groups) on body weight ($P = 0.797$) or lung volume ($P = 0.326$). OVA challenge (i.e., Allergy groups) also had no effect on body weight ($P = 0.588$) or lung volume ($P = 0.918$).

Airway responsiveness: Prior to MCh challenge (post-saline), R_n was higher in the presence of Remodelling ($P = 0.009$, $n = 9$; Figure 2) but not Allergy ($P = 0.372$, $n = 11$; Figure 2), compared with the Control group. R_n increased with increasing doses of MCh in all groups (Figure 2). At the maximal dose of MCh, responses were significantly increased in the Allergy ($P = 0.006$; Figure 2) and Remodelling ($P < 0.001$; Figure 2) groups compared with the Control group ($n = 9$). The ΔR_n was also increased in the presence of Allergy ($P = 0.007$) and Remodelling ($P < 0.001$) groups compared with Control. In the Allergy and Remodelling group, R_n was greater than either allergy or remodelling alone ($P < 0.05$, $n = 13$; Figure 2). Since there was no significant interaction between allergy and remodelling, the effects of allergy and remodelling are likely to be additive. There were no significant differences in G between groups (Figure 3A). The change in H after MCh was however increased in the presence of Remodelling ($P = 0.014$) compared with Control (Figure 3B).

Airway morphometry and histology: As intended, compared with the Control group (Figure 4 and 5A, Table 2), the thickness of the ASM layer was increased in the groups that were exposed to doxycycline i.e., Remodelling, or Allergy and Remodelling ($P = 0.028$; Figure 4, 4, 5C and 5D, Table 2) groups. WA_i was also increased in the Remodelling groups ($P = 0.047$; Table 2). However, there were no changes in any other airway wall parameter. There was no change in airway wall structure in the Allergy group compared with the Control

group (Figure 4 and 5B, Table 2). Multiple linear regression showed positive associations between ASM thickness and ΔR_n (slope $P = 0.003$; Figure 6A) and the relationship was upward shifted in the presence of allergy (intercept $P = 0.012$). That is, in the Allergy groups, there was a greater increase in R_n for any given thickness of ASM. These associations were parallel with no effect of allergy on the slope of the $\Delta R_n - \text{ASM}$ relationship ($P = 0.99$). Similar relationships were observed between WA_i and ΔR_n (Figure 6B).

Compared with Control, mucin expression was increased in the Remodelling groups ($P = 0.049$) and the Allergy groups ($P < 0.001$). Mucin expression was $5.0 \pm 1.9\%$ in the Control group ($n = 8$), $17.5 \pm 5.6\%$ in the Allergy group ($n = 9$), $9.3 \pm 1.8\%$ in the Remodelling group ($n = 10$), and $26.7 \pm 4.8\%$ in the Allergy and Remodelling group ($n = 16$). The presence of allergy did not modify the association between mucin expression and remodelling.

Total IgE was detected in the serum of all groups (Table 3). Total serum IgE was greater in the presence of remodelling, indicating an independent effect of doxycycline ($P = 0.016$). Multiple linear regression analysis showed no relationship between IgE and ΔR_n ($P = 0.89$). The semi-quantitative inflammatory score was similar between treatment groups ($P = 0.249$). Two mice from the Remodelling group ($n = 10$), and one mouse from the Allergy and Remodelling group ($n = 16$) had mild fibrosis. One mouse in the Allergy and Remodelling group had moderate fibrosis. No fibrosis was observed in other mice.

DISCUSSION

In this study, we used a transgenic mouse model of increased ASM thickness (without inflammation) and an OVA-induced allergy model (without remodelling) to show that

increased ASM thickness and allergy contribute independently and additively to AHR. These observations support an alternative paradigm to animal models of asthma which require repeated aeroallergen exposure to produce thickening of the ASM layer (27) and have in turn been used as evidence to support the hypothesis that allergen/inflammation drives the remodelling process. The present study now shows that the same phenotype of allergy and remodelling can be produced by separate susceptibilities (or simulations in the context of an experiment) to morphological and inflammatory abnormalities. Moreover, a common criticism of conventional mouse models (e.g., OVA) is that the level of 'AHR' (increased ΔR_n) is far less severe than that observed in asthmatic patients (28). It was however previously demonstrated that airway responsiveness in OVA exposed mice could be increased when combined with protease-mediated destruction of the airway epithelial barrier, supporting a theoretical multiple-hit model of AHR (29). Informed by clinical studies on the trajectory of airway pathology in asthma, notably the presence of ASM thickening early in the clinical course of the disease (1, 11) and which is potentially independent of inflammation (30), we observed that the effects of increased ASM thickness and allergy on ΔR_n is greater than that which could be achieved by either allergy or remodelling alone.

The primary structural and functional abnormalities associated with asthma are increased thickness of the ASM layer and AHR. Allergic asthma is the most common form of the disease and there is debate whether allergic airway inflammation drives airway wall remodelling or combines with existing remodelling (due to other causes) to produce AHR. In asthmatic patients, ASM thickness is increased early in life (11) and appears unrelated to the duration of disease (1). If allergic inflammation drives the remodelling process, then ASM thickness in asthmatics should increase progressively throughout life. Although this is seen to some extent in mild-moderate cases of asthma, ASM thickness seems largely determined

early in life. This is consistent with epidemiological studies showing that deficits in lung function occur early in life and any decline in lung function in later life differs only marginally from the healthy individuals (31-33). The relatively stable discrepancy in lung function between subjects with and without asthma is matched by stability of the clinical severity of asthma (34).

We, and others, hypothesize that structural airway wall abnormalities and airway inflammation (driven by allergy) observed in asthma might have independent origins. The observations of Cockcroft et al. (35) that the response to an inhaled allergen is independently related to non-specific AHR to MCh as well as to the skin prick test response to the same allergen highlights the possible independent effects of remodelling and the inflammatory response. Various abnormalities that are characteristic of asthma may develop as a result of separate genetic susceptibilities (36). While some individuals may be genetically predisposed to develop AHR, and others predisposed to allergy, it is perhaps the individuals with multiple predispositions who ultimately develop asthma. This theory could account for the group of individuals who exhibit AHR but have no past or present history of asthma i.e., asymptomatic AHR (37, 38). Separate origins of inflammation and remodelling could also contribute to within and between patient variability in disease severity (39, 40).

To determine the independent effect of increased ASM thickness we used a sophisticated transgenic mouse model where the remodelling develops independently of inflammation (17). The remodelling process was initiated by the doxycycline mediated overexpression of TGF- α . The signalling pathways that promote ASM thickening in asthma are unclear but may well include the actions of growth factors such as TGF- α that bind to epidermal growth factor receptor (EGFR). In subjects with asthma, EGFR expression is increased in the epithelium

and correlates with the magnitude of sub-epithelial reticular membrane thickness (41). Similarly, expression of ligands for EGFR including TGF- α is increased in ASM cells and epithelial cells from asthmatic patients (42). Morphometric analyses in the present study demonstrated an increase in the thickness of the ASM layer without evidence of inflammation, consistent with previous studies (15, 17).

To determine the independent effect of allergy on airway responsiveness, we used the conventional OVA model. The OVA induced model of allergic airway disease has been useful for studying mechanisms relating to increased responsiveness to bronchoconstricting agents (18). Once systemic sensitisation to OVA has occurred, a single exposure to inhaled OVA produces an inflammatory cellular influx peaking 6 h post-challenge and returning to normal levels 8 h post-challenge (18), while AHR remains elevated 24 h after challenge (18). These observations are consistent with that of the present study where there was an independent effect of OVA (i.e., allergy) on AHR even though the inflammatory cellular influx had subsided. Most importantly, there was no evidence of airway remodelling, particularly increased ASM thickness, which was expected since the exposure was limited to a single allergen challenge.

The combined effects of remodelling and allergy may be additive or synergistic. However, the lack of a significant interaction term in our two-way ANOVA suggests that the effects of increased ASM thickness and allergy on airway responsiveness were independent and additive. While this does not necessarily rule out a synergistic effect of remodelling and allergy, at the very least there does not seem to be strong evidence of synergy. Additive effects of remodelling and allergy are consistent with the data shown in Figure 6A which shows the correlations between ASM thickness and ΔR_n with and without allergy. The

thickness of the ASM layer was positively correlated with ΔR_n and this relationship was upward shifted in the presence of allergy (change in Y intercept) without an effect on the slope of the relationship. One possible interpretation of these data is that the differences between these linear plots reflect changes in ASM contractility in the presence of inflammatory stimuli which by definition increases ASM force for a given ASM thickness (43). However, an inherent assumption is that, under each condition (allergy or not), MCh produces the same level of ASM activation, which may not be correct since epithelial permeability can increase in allergic disease (44) or alternatively decrease due to the presence of mucus deposition (45).

If changes in ASM thickness in asthma arise early in life and are determined separately from inflammation, this raises the question as to how an excess in ASM mass develops (46). A developmental origin of increased ASM thickness is plausible, and there is evidence that developmental disorders such as intrauterine growth restriction (IUGR) are associated with asthma (47). The reason for the association between abnormal fetal growth and asthma is not known but could involve structural changes to the airway wall (48). For instance, fetal hypoxia is a common cause of IUGR and the proliferative capacity of fetal ASM cells is enhanced under conditions of hypoxia (49). Preventing excessive ASM growth early in life may represent a novel intervention to reduce the development of asthma in high risk individuals. Once we better understand the independent role of increased ASM thickness in asthma pathogenesis, appropriate early life prevention strategies can be considered to reduce the burden of asthma.

We chose to focus on increased thickness of the ASM layer as this contributes substantially to the overall increase in airway wall thickness in patients with asthma (14). Non-muscle

remodelling is also reported in asthma (50) and is predicted in mathematical models to exaggerate the airway narrowing response independent of changes in ASM force production (51). Increased WA_i was observed in the Remodelling groups, and there was a relationship between ΔR_n and WA_i , similar to that observed with ASM thickness. For a given level of ASM shortening, increased WA_i geometrically favours an increase in lumen narrowing (52). A similar geometric effect on ΔR_n might also be predicted due to the release of mucus, which effectively increases WA_i . Measurement of mucus release is technically difficult, but can be indirectly assessed from the proportion of mucin within the epithelial layer. Mucin expression was increased in the Allergy groups which is expected (53) and also in the Remodelling groups. To our knowledge, an effect of TGF- α on epithelial (goblet cell) mucin expression has not previously been demonstrated. Admittedly, increased mucin expression does not definitely translate into increase mucus within the airway lumen, though this seems likely. The implications are that increases in airway resistance with MCh are partly mediated by mucus release. Airway closure due to mucus plugging may in turn increase H, since isolation of alveoli units reduces the effective lung size. The increase in H after MCh was greater in the Remodelling groups, but there was no change in H in the Allergy groups. Nonetheless, the direct and indirect effects of mucus release on lung function should not be ignored.

While our proposal is that changes in lung function are due to independent effects of airway remodelling and allergy, doxycycline and OVA may act through other pathways. With more extended durations of doxycycline (3-6 weeks), body weight is reduced (26) and lung volume increased (54), reflecting more chronic respiratory disease including fibrosis (17). In comparison, since doxycycline exposure was limited to 10 d in the present study, there was very limited evidence of fibrosis and no independent effect of doxycycline exposure on either

lung volume or body weight. Exposure to doxycycline increased serum IgE raising the possibility that the overall increase in ΔR_n in the Allergy and Remodelling group was due to a greater inflammatory response to OVA. There was no evidence of an increased inflammatory response to OVA at the time of physiological measurement (24 h post-OVA). Furthermore, if IgE enhanced the inflammatory response to OVA, this would favour a synergistic interaction, and as discussed, there was no strong evidence of synergy. Finally, since there was an independent effect of doxycycline on IgE levels, some of the increase in ΔR_n could be mediated by an increase ASM contractility (55) opposed to a structure-dependent mechanism. This possibility seems unlikely given our previous published data demonstrating a clear association between ASM thickness and AHR in unsensitised transgenic mice (15) and the lack of association between IgE and ΔR_n in regression analyses.

In summary, our findings challenge the current paradigm of asthma pathogenesis and support independent contributions of allergy and airway remodelling in asthma that may be determined by separate genetic and/or environmental susceptibilities. Decoupling of these key abnormalities in asthma has implications for the treatment and prevention of disease.

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DECLARATION OF INTEREST

None.

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AUTHORS CONTRIBUTION

K.C.W.W., T.D.L.C., A.N.L., G.R.Z., A.L.J. and P.B.N. designed the study; K.C.W.W. performed all experiments and generated the mouse colony; K.C.W.W. and J.G.E. were involved in data collection; K.C.W.W., A.N.L., G.R.Z. and P.B.N. analysed the data; K.C.W.W., A.L.J. and P.B.N. drafted the manuscript; all authors contributed to data interpretation and approved the final manuscript.

REFERENCES

1. James AL, Bai TR, Mauad T, Abramson MJ, Dolhnikoff M, McKay KO, et al. Airway smooth muscle thickness in asthma is related to severity but not duration of asthma. *Eur Respir J*. 2009;34(5):1040-5.
2. Noble PB, Jones RL, Cairncross A, Elliot JG, Mitchell HW, James AL, et al. Airway narrowing and bronchodilation to deep inspiration in bronchial segments from subjects with and without reported asthma. *J Appl Physiol* (1985). 2013;114(10):1460-71.
3. Wardlaw AJ, Brightling C, Green R, Woltmann G, Pavord I. Eosinophils in asthma and other allergic diseases. *Br Med Bull*. 2000;56(4):985-1003.
4. Pavord ID. Non-eosinophilic asthma and the innate immune response. *Thorax*. 2007;62(3):193-4.

- 449 5. Simpson BM, Custovic A, Simpson A, Hallam CL, Walsh D, Marolia H, et al. NAC
450 Manchester Asthma and Allergy Study (NACMAAS): risk factors for asthma and allergic
451 disorders in adults. *Clin Exp Allergy*. 2001;31(3):391-9.
- 452 6. Illi S, von Mutius E, Lau S, Nickel R, Niggemann B, Sommerfeld C, et al. The pattern
453 of atopic sensitization is associated with the development of asthma in childhood. *J Allergy*
454 *Clin Immunol*. 2001;108(5):709-14.
- 455 7. Elliot JG, Jones RL, Abramson MJ, Green FH, Mauad T, McKay KO, et al.
456 Distribution of airway smooth muscle remodelling in asthma: relation to airway
457 inflammation. *Respirology*. 2015;20(1):66-72.
- 458 8. Kicic A, Sutanto EN, Stevens PT, Knight DA, Stick SM. Intrinsic biochemical and
459 functional differences in bronchial epithelial cells of children with asthma. *Am J Respir Crit*
460 *Care Med*. 2006;174(10):1110-8.
- 461 9. Hackett TL, Warner SM, Stefanowicz D, Shaheen F, Pechkovsky DV, Murray LA, et
462 al. Induction of epithelial-mesenchymal transition in primary airway epithelial cells from
463 patients with asthma by transforming growth factor-beta1. *Am J Respir Crit Care Med*.
464 2009;180(2):122-33.
- 465 10. Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-
466 cell infiltration of airway smooth muscle in asthma. *N Engl J Med*. 2002;346(22):1699-705.
- 467 11. O'Reilly R, Ullmann N, Irving S, Bossley CJ, Sonnappa S, Zhu J, et al. Increased
468 airway smooth muscle in preschool wheezers who have asthma at school age. *J Allergy Clin*
469 *Immunol*. 2013;131(4):1024-32.
- 470 12. Haahtela T. Advances in pharmacotherapy of asthma. *Curr Probl Dermatol*.
471 1999;28:135-52.

- 472 13. Pretolani M, Bergqvist A, Thabut G, Dombret MC, Knapp D, Hamidi F, et al.
473 Effectiveness of bronchial thermoplasty in patients with severe refractory asthma: Clinical
474 and histopathologic correlations. *J Allergy Clin Immunol.* 2017;139(4):1176-85.
- 475 14. James AL, Wenzel S. Clinical relevance of airway remodelling in airway diseases.
476 *Eur Respir J.* 2007;30(1):134-55.
- 477 15. Wang KCW, Astell CJ, Wijesinghe P, Larcombe AN, Pinniger GJ, Zosky GR, et al.
478 Optical coherence tomography-based contact indentation for diaphragm mechanics in a
479 mouse model of transforming growth factor alpha induced lung disease. *Sci Rep.*
480 2017;7(1):1517.
- 481 16. Perl AK, Zhang L, Whitsett JA. Conditional expression of genes in the respiratory
482 epithelium in transgenic mice: cautionary notes and toward building a better mouse trap. *Am*
483 *J Respir Cell Mol Biol.* 2009;40(1):1-3.
- 484 17. Kramer EL, Mushaben EM, Pastura PA, Acciani TH, Deutsch GH, Khurana Hershey
485 GK, et al. Early growth response-1 suppresses epidermal growth factor receptor-mediated
486 airway hyperresponsiveness and lung remodeling in mice. *Am J Respir Cell Mol Biol.*
487 2009;41(4):415-25.
- 488 18. Zosky GR, Larcombe AN, White OJ, Burchell JT, Janosi TZ, Hantos Z, et al.
489 Ovalbumin-sensitized mice are good models for airway hyperresponsiveness but not acute
490 physiological responses to allergen inhalation. *Clin Exp Allergy.* 2008;38(5):829-38.
- 491 19. Larcombe AN, Foong RE, Bozanich EM, Berry LJ, Garratt LW, Gualano RC, et al.
492 Sexual dimorphism in lung function responses to acute influenza A infection. *Influenza Other*
493 *Respi Viruses.* 2011;5(5):334-42.
- 494 20. Janosi TZ, Adamicza A, Zosky GR, Asztalos T, Sly PD, Hantos Z. Plethysmographic
495 estimation of thoracic gas volume in apneic mice. *J Appl Physiol.* 2006;101:454-9.

- 496 21. Bozanich EM, Collins RA, Thamrin C, Hantos Z, Sly PD, Turner DJ. Developmental
497 changes in airway and tissue mechanics in mice. *J Appl Physiol* (1985). 2005;99(1):108-13.
- 498 22. Collins RA, Gualano RC, Zosky GR, Atkins CL, Turner DJ, Colasurdo GN, et al.
499 Hyperresponsiveness to inhaled but not intravenous methacholine during acute respiratory
500 syncytial virus infection in mice. *Respir Res*. 2005;6:142.
- 501 23. Hantos Z, Daróczy B, Suki B, Nagy S, Fredberg JJ. Input impedance and peripheral
502 inhomogeneity of dog lungs. *J Appl Physiol* (1985). 1992;72(1):168-78.
- 503 24. Cheah EY, Mann TS, Burcham PC, Henry PJ. Functional characterisation and
504 application of an ex vivo perfusion-superfusion system in murine airways. *J Pharmacol*
505 *Toxicol Methods*. 2016;84:66-77.
- 506 25. James AL, Hogg JC, Dunn LA, Paré PD. The use of the internal perimeter to compare
507 airway size and to calculate smooth muscle shortening. *Am Rev Respir Dis*.
508 1988;138(1):136-9.
- 509 26. Hardie WD, Le Cras TD, Jiang K, Tichelaar JW, Azhar M, Korfhagen TR.
510 Conditional expression of transforming growth factor-alpha in adult mouse lung causes
511 pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol*. 2004;286(4):L741-9.
- 512 27. McMillan SJ, Lloyd CM. Prolonged allergen challenge in mice leads to persistent
513 airway remodelling. *Clin Exp Allergy*. 2004;34(3):497-507.
- 514 28. Wenzel S, Holgate ST. The mouse trap: It still yields few answers in asthma. *Am J*
515 *Respir Crit Care Med*. 2006;174(11):1173-6.
- 516 29. Bates JH, Cojocaru A, Haverkamp HC, Rinaldi LM, Irvin CG. The synergistic
517 interactions of allergic lung inflammation and intratracheal cationic protein. *Am J Respir Crit*
518 *Care Med*. 2008;177(3):261-8.

- 519 30. Lezmi G, Gosset P, Deschildre A, Abou-Taam R, Mahut B, Beydon N, et al. Airway
520 Remodeling in Preschool Children with Severe Recurrent Wheeze. *Am J Respir Crit Care*
521 *Med.* 2015;192(2):164-71.
- 522 31. Phelan PD, Robertson CF, Olinsky A. The Melbourne Asthma Study: 1964-1999. *J*
523 *Allergy Clin Immunol.* 2002;109(2):189-94.
- 524 32. James AL, Palmer LJ, Kicic E, Maxwell PS, Lagan SE, Ryan GF, et al. Decline in
525 lung function in the Busselton Health Study: the effects of asthma and cigarette smoking. *Am*
526 *J Respir Crit Care Med.* 2005;171(2):109-14.
- 527 33. Sears MR, Greene JM, Willan AR, Wiecek EM, Taylor DR, Flannery EM, et al. A
528 longitudinal, population-based, cohort study of childhood asthma followed to adulthood. *N*
529 *Engl J Med.* 2003;349(15):1414-22.
- 530 34. Horak E, Lanigan A, Roberts M, Welsh L, Wilson J, Carlin JB, et al. Longitudinal
531 study of childhood wheezy bronchitis and asthma: outcome at age 42. *BMJ.*
532 2003;326(7386):422-3.
- 533 35. Cockcroft DW, Davis BE, Boulet LP, Deschesnes F, Gauvreau GM, O'Byrne PM, et
534 al. The links between allergen skin test sensitivity, airway responsiveness and airway
535 response to allergen. *Allergy.* 2005;60(1):56-9.
- 536 36. Melén E, Pershagen G. Pathophysiology of asthma: lessons from genetic research
537 with particular focus on severe asthma. *J Intern Med.* 2012;272(2):108-20.
- 538 37. Jones A. Asymptomatic bronchial hyperreactivity and the development of asthma and
539 other respiratory tract illnesses in children. *Thorax.* 1994;49(8):757-61.
- 540 38. Laprise C, Boulet LP. Asymptomatic airway hyperresponsiveness: a three-year
541 follow-up. *Am J Respir Crit Care Med.* 1997;156(2 Pt 1):403-9.
- 542 39. Saglani S, Lloyd CM. Novel concepts in airway inflammation and remodelling in
543 asthma. *Eur Respir J.* 2015;46(6):1796-804.

- 544 40. Jeffery PK. Remodeling in asthma and chronic obstructive lung disease. *Am J Respir*
545 *Crit Care Med.* 2001;164(10 Pt 2):S28-38.
- 546 41. Puddicombe SM, Polosa R, Richter A, Krishna MT, Howarth PH, Holgate ST, et al.
547 Involvement of the epidermal growth factor receptor in epithelial repair in asthma. *FASEB J.*
548 2000;14(10):1362-74.
- 549 42. Lordan JL, Bucchieri F, Richter A, Konstantinidis A, Holloway JW, Thornber M, et
550 al. Cooperative effects of Th2 cytokines and allergen on normal and asthmatic bronchial
551 epithelial cells. *J Immunol.* 2002;169(1):407-14.
- 552 43. Ansell TK, Mitchell HW, McFawn PK, Noble PB. TNF and IL-1 β exposure increases
553 airway narrowing but does not alter the bronchodilatory response to deep inspiration in
554 airway segments. *Respirology.* 2016;21(6):1041-8.
- 555 44. Xiao C, Puddicombe SM, Field S, Haywood J, Broughton-Head V, Puxeddu I, et al.
556 Defective epithelial barrier function in asthma. *J Allergy Clin Immunol.* 2011;128(3):549-
557 56.e1-12.
- 558 45. Bates JH, Stevenson CA, Aliyeva M, Lundblad LK. Airway responsiveness depends
559 on the diffusion rate of methacholine across the airway wall. *J Appl Physiol* (1985).
560 2012;112(10):1670-7.
- 561 46. Stewart A. More muscle in asthma, but where did it come from? *Am J Respir Crit*
562 *Care Med.* 2012;185(10):1035-7.
- 563 47. Källén B, Finnström O, Nygren KG, Otterblad Olausson P. Association between
564 preterm birth and intrauterine growth retardation and child asthma. *Eur Respir J.*
565 2013;41(3):671-6.
- 566 48. Wang KCW, Morton JS, Davidge ST, Larcombe AN, James AL, Donovan GM, et al.
567 Increased heterogeneity of airway calibre in adult rats after hypoxia-induced intrauterine
568 growth restriction. *Respirology.* 2017;22(7):1329-35.

49. Hartman WR, Smelter DF, Sathish V, Karass M, Kim S, Aravamudan B, et al. Oxygen dose responsiveness of human fetal airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol.* 2012;303(8):L711-9.
50. Carroll N, Elliot J, Morton A, James A. The structure of large and small airways in nonfatal and fatal asthma. *Am Rev Respir Dis.* 1993;147(2):405-10.
51. Lambert RK, Paré PD. Lung parenchymal shear modulus, airway wall remodeling, and bronchial hyperresponsiveness. *J Appl Physiol* (1985). 1997;83(1):140-7.
52. 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* (1985). 2004;96(6):2019-27.
53. Singer M, Martin LD, Vargaftig BB, Park J, Gruber AD, Li Y, et al. A MARCKS-related peptide blocks mucus hypersecretion in a mouse model of asthma. *Nat Med.* 2004;10(2):193-6.
54. Le Cras TD, Hardie WD, Deutsch GH, Albertine KH, Ikegami M, Whitsett JA, et al. Transient induction of TGF- α disrupts lung morphogenesis, causing pulmonary disease in adulthood. *Am J Physiol Lung Cell Mol Physiol.* 2004;287(4):L718-29.
55. Watson N, Bodtke K, Coleman RA, Dent G, Morton BE, Rühlmann E, et al. Role of IgE in hyperresponsiveness induced by passive sensitization of human airways. *Am J Respir Crit Care Med.* 1997;155(3):839-44.

FIGURE LEGENDS

Figure 1. Sensitization, diet, and protocols for Control, Allergy, Remodelling, and Allergy and Remodelling groups. OVA, ovalbumin.

Figure 2. Changes in R_n with MCh challenge. Control (closed circles); Allergy (grey squares), Remodelling (open circles), Allergy and Remodelling groups (grey circles). Allergy and Remodelling independently increased maximal R_n and ΔR_n . The combined effects of allergy and remodelling produced a greater increase in R_n than either allergy or remodelling alone. # Significantly different from Control ($P < 0.05$); ^ Significantly different from Allergy ($P < 0.05$); & Significantly different from Remodelling ($P < 0.05$). R_n , Newtonian resistance.

Figure 3. Changes in G (A) and H (B) with MCh challenge. Control (closed circles); Allergy (grey squares), Remodelling (open circles), Allergy and Remodelling groups (grey circles). G, tissue damping; H, tissue elastance. # Significantly different from Control ($P < 0.05$); ^ Significantly different from Allergy ($P < 0.05$).

Figure 4. Thickness of the ASM layer. * Doxycycline exposure had an independent effect on ASM thickness ($P < 0.05$) and therefore ASM thickness was greater in the Remodelling groups. ASM, airway smooth muscle; P_{bm} , perimeter of basement membrane.

Figure 5. Representative images of airways from Control (A), Allergy (B), Remodelling (C) and Allergy and Remodelling (D) groups ($\times 100$). ASM, airway smooth muscle.

613 **Figure 6.** Relationship between ΔR_n in response to MCh and the thickness of the ASM layer
614 (A) in the presence of allergy (grey dotted line and closed circles) and in the absence of
615 allergy (black solid line and closed circles). In the presence of allergy, the relationship was
616 upward shifted with no effect on slope. There were similar associations between ΔR_n and
617 WA_i (B). ASM, airway smooth muscle; R_n , Newtonian resistance; ΔR_n , change in R_n after
618 MCh (post MCh R_n – post saline R_n); P_{bm} , perimeter of basement membrane; WA_i , inner
619 airway wall.

620 **TABLES**621 **Table 1.** Group characteristics.

	Control	Allergy	Remodelling	Allergy and Remodelling
Sex distribution (male:female)	5:5	5:6	5:5	7:9
Body weights (g)	20.32 ± 1.06 (10)	20.48 ± 0.87 (11)	19.83 ± 0.93 (10)	20.44 ± 0.57 (16)
Lung volume (mL)	0.36 ± 0.04 (9)	0.33 ± 0.04 (11)	0.35 ± 0.03 (10)	0.40 ± 0.04 (15)

622 Values are mean ± SEM (n).

623

624 **Table 2.** Airway dimensions.

	Control (n=10)	Allergy (n=10)	Remodelling (n=10)	Allergy and Remodelling (n=16)
P _{bm} (mm)	3.03 ± 0.27	2.99 ± 0.32	2.833 ± 0.15	2.69 ± 0.16
ASM ($\sqrt{\text{area}/P_{bm}}$)	0.036 ± 0.003	0.036 ± 0.003	0.043 ± 0.002*	0.045 ± 0.004*
WA _t ($\sqrt{\text{area}/P_{bm}}$)	0.15 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.14 ± 0.01
WA _o ($\sqrt{\text{area}/P_{bm}}$)	0.13 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.10 ± 0.01
WA _i ($\sqrt{\text{area}/P_{bm}}$)	0.06 ± 0.005	0.07 ± 0.01	0.07 ± 0.004*	0.09 ± 0.01*
Lumen ($\sqrt{\text{area}/P_{bm}}$)	0.21 ± 0.01	0.22 ± 0.01	0.19 ± 0.01	0.21 ± 0.01
Epithelium ($\sqrt{\text{area}/P_{bm}}$)	0.05 ± 0.004	0.054 ± 0.004	0.06 ± 0.003	0.06 ± 0.01

625 Values are mean $\pm$ SEM. *Doxycycline effect ($P < 0.05$). ASM, airway smooth muscle; WA_i ,
 626 inner airway wall; WA_o , outer airway wall; WA_t , total airway wall; P_{bm} , perimeter of
 627 basement membrane.

628

629 **Table 3.** IgE levels.

	Control (n=9)	Allergy (n=9)	Remodelling (n=8)	Allergy and Remodelling (n=10)
Total serum IgE (ng/mL)	1206 $\pm$ 427	1557 $\pm$ 389	1972 $\pm$ 300*	2383 $\pm$ 201*

630 Values are mean $\pm$ SEM. *Doxycycline effect ($P < 0.05$).











