ORIGINAL ARTICLE

The activin A antagonist follistatin inhibits asthmatic airway remodelling

Charles Linton Hardy,[1,2,3] Hong-An Nguyen,[1,2,3] Rohimah Mohamud,[1,3] John Yao,[1,2,3] Ding Yuan Oh,[1,2] Magdalena Plebanski,[1,3] Kate L Loveland,[4] Craig A Harrison,[5] Jennifer M Rolland,[1,2,3] Robyn E O'Hehir[1,2,3]

▶ Additional supplementary files are published online only. To view these files please visit the journal online (http://dx.doi.org/10.1136/thoraxjnl-2011-201128).

[1]Department of Immunology, Monash University, Melbourne, Victoria, Australia
[2]Department of Allergy, Immunology and Respiratory Medicine, Monash University and The Alfred Hospital, Melbourne, Victoria, Australia
[3]CRC for Asthma and Airways, Sydney, Australia
[4]Department of Biochemistry, Molecular Biology, Anatomy and Developmental Biology, School of Biomedical Sciences, Monash University, Clayton, Victoria, Australia
[5]Growth Factor Signaling, Prince Henry's Institute, Clayton, Victoria, Australia

Correspondence to
Dr Charles Linton Hardy, Department of Immunology, Monash University, 89 Commercial Road, Level 2, Melbourne, VIC 3004, Australia; charles.hardy@monash.edu

Received 19 September 2011
Revised 26 August 2012
Accepted 30 August 2012
Published Online First 10 October 2012

To cite: Hardy CL, Nguyen H-A, Mohamud R, *et al*. *Thorax* 2013, **68**, 9–18.

ABSTRACT

Background Current pharmacotherapy is highly effective in the clinical management of the majority of patients with stable asthma, however severe asthma remains inadequately treated. Prevention of airway remodelling is a major unmet clinical need in the management of patients with chronic severe asthma and other inflammatory lung diseases. Accumulating evidence convincingly demonstrates that activin A, a member of the transforming growth factor (TGF)-β superfamily, is a key driver of airway inflammation, but its role in chronic asthmatic airway remodelling is ill-defined. Follistatin, an endogenously produced protein, binds activin A with high affinity and inhibits its bioactivity. The aim of this study was to test the potential of follistatin as a therapeutic agent to inhibit airway remodelling in an experimental model of chronic allergic airway inflammation.

Methods BALB/c mice were systemically sensitised with ovalbumin (OVA), and challenged with OVA intranasally three times a week for 10 weeks. Follistatin was instilled intranasally during allergen challenge.

Results Chronic allergen challenge induced mucus hypersecretion and subepithelial collagen deposition which persisted after cessation of challenge. Intranasal follistatin (0.05, 0.5, 5 μg) inhibited the airway remodelling and dose-dependently decreased airway activin A and TGF-β1, and allergen-specific T helper 2 cytokine production in the lung-draining lymph nodes. Follistatin also impaired the loss of TGF-β1 and activin RIB immunostaining in airway epithelium which occurred following chronic allergen challenge.

Conclusions These data demonstrate that follistatin attenuates asthmatic airway remodelling. Our findings point to the potential of follistatin as a therapeutic for prevention of airway remodelling in asthma and other inflammatory lung diseases.

Key messages

What is the key question?
- To determine whether blocking activin A with its naturally occurring antagonist follistatin inhibits asthmatic airway remodelling.

What is the bottom line?
- Follistatin instillation during allergen challenge inhibited secretion of activin A and transforming growth factor β1 in the lung, and significantly inhibited subepithelial collagen deposition and airway epithelial mucus production.

Why read on?
- Our findings provide insight into the therapeutic potential of follistatin in the control of fibrosis in lung inflammatory disease, and highlight a role for activin A in the regulation of inflammation.

INTRODUCTION

Current pharmacotherapy is highly effective in the clinical management of the majority of patients with stable asthma, however severe asthma remains inadequately treated. Prevention of airway remodelling is a major unmet clinical need in the management of patients with chronic severe asthma and other inflammatory lung diseases.[1] Increasing evidence implicates activin A, a homodimer of activin βA subunits and a member of the transforming growth factor (TGF)-β superfamily, in this process. Activin A can promote inflammation by stimulating production of inflammatory mediators including interleukin (IL)-1β, IL-6, tumour necrosis factor and nitric oxide. However, other studies show that activin A can inhibit inflammation.[2 3] Clearly, regulation of the immunoregulatory effect of activin A is complex and dependent on the anatomical site, cell type and phase of the immune response. Importantly, activin A bioactivity is inhibited by the endogenously produced high-affinity binding protein follistatin.[4]

Accumulating data suggest that activin A regulates inflammation and fibrosis in the lung.[5–9] In vitro, activin A stimulates proliferation of human lung fibroblasts and smooth muscle cells.[5 10] In vivo, activin A is upregulated in bleomycin-induced pulmonary fibrosis, and follistatin injection inhibits this fibrosis.[11] Furthermore, mice deficient in Smad3, common to the TGF-β/activin A signalling pathways, have decreased airway remodelling.[12] In contrast, overexpression of the TGF-β/activin signalling intermediate Smad2 in airway epithelium induced activin A secretion and airway remodelling.[13] Remodelling was inhibited by injection of activin A neutralising antibody prior to allergen challenge, providing direct evidence of a role for

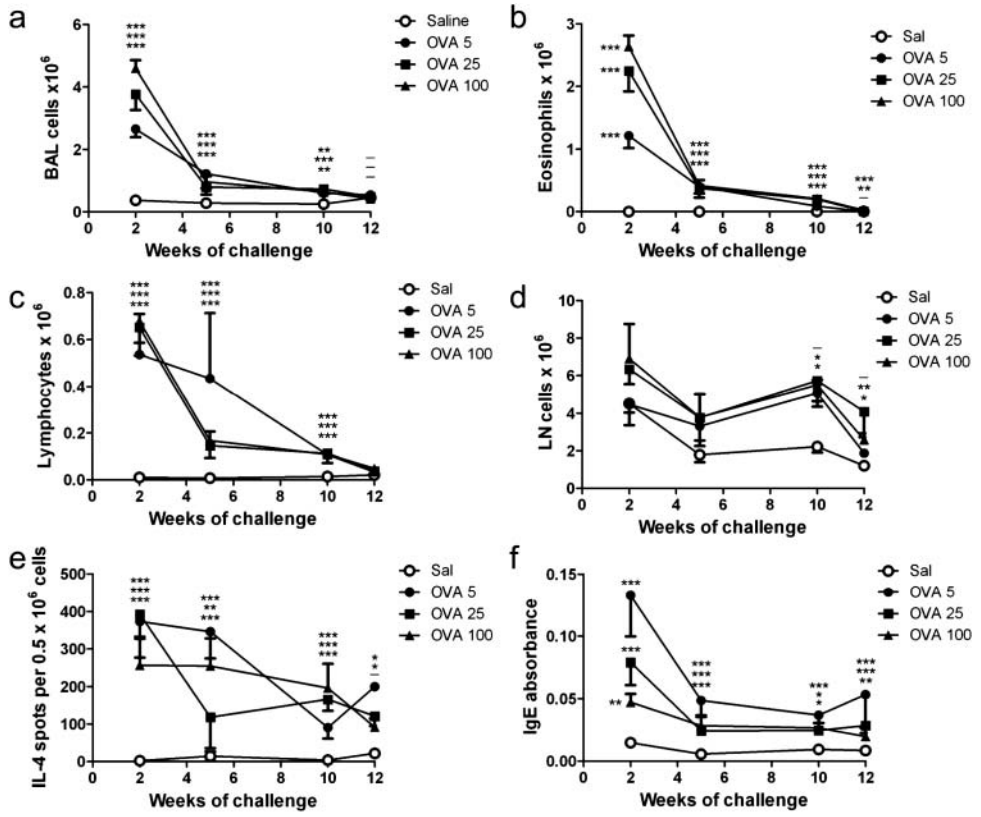


Figure 1 Chronic allergen challenge causes resolution of allergic inflammation independent of allergen challenge dose. Mice were sensitised and challenged with OVA as per online supplementary figure S1 A; controls received saline instead of OVA. (A–D) Counts of total bronchoalveolar lavage (BAL) cells, BAL eosinophils, BAL lymphocytes, and draining LN cells, and (E, F) frequency of interleukin (IL)-4-producing cells in the lung-draining LN, and levels of serum OVA-specific IgE were measured at the time points shown in (A). Mean ± SEM, n = 6–7 mice/group per time point. *p<0.05, **p<0.01, ***p<0.001; the order of symbols from top to bottom is OVA 5, 25 and 100 μg (relative to saline).

activin A in remodelling. However, detailed knowledge of the effect of inhibiting activin A with follistatin in chronic asthma is lacking. In the current study we investigated whether follistatin treatment during allergen challenge inhibited development of airway remodelling in a model of chronic allergic asthma. Our data identify activin A as an important driver of asthmatic airway pathology and highlight the therapeutic potential of follistatin as an inhibitor of airway remodelling.

METHODS

Mice

Female BALB/c mice (7 weeks) were obtained from the Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria, Australia and housed in the Alfred Medical Research and Education Precinct (AMREP) animal house. All experimental protocols were approved by the AMREP Animal Ethics Committee.

Recombinant follistatin and follistatin luciferase bioassay

For follistatin production and assessment of bioactivity, see online supplement.

Immunisations and tissue processing

Mice were sensitised with intraperitoneal ovalbumin (OVA; A5503, Sigma-Aldrich, Saint Louis, Missouri, USA) in alum,[6] and isofluorane-anaesthetised mice challenged intranasally (50 μl) with OVA (5 μg, 25 μg or 100 μg) three times per week.[14–16] Groups were killed after 2, 5 or 10 weeks of allergen challenge, and 2 weeks after the final challenge (12 weeks) (see online supplementary figure S1A). For the follistatin instillation experiments, OVA-sensitised mice were challenged intranasally with OVA (25 μg) alone or mixed with follistatin (0.05, 0.5 or 5 μg), and analysis performed after 5 weeks of challenge (see online supplementary figure S1B). Controls received saline instead of OVA or follistatin. Bronchoalveolar lavage (BAL), differentials and tissue sampling were as described[17] (see online supplement).

Cytokine ELISPOT, OVA-specific IgE, activin A, TGF-β1 and IL-13 ELISA

IL-4, IL-5 and IL-13 enzyme-linked immunosorbent spot (ELISPOT) on lung-draining lymph node (LN) cells were performed as described.[6] OVA-specific IgE was detected as described.[17] Activin A was measured by a specific ELISA as described.[6 18] TGF-β1 was detected using a TGF-β1 ELISA kit (#DY1679, R&D Systems, Minneapolis, MN, USA) (see online supplement). IL-13 was detected using the IL-13 Ready-SET-Go! kit (#88-7137-88; eBioscience, Inc., San Diego, California, USA).

Follistatin radioimmunoassay

Follistatin concentrations were measured using a discontinuous radioimmunoassay as described.[18]

Activin A, TGF-β, follistatin and activin receptor immunohistochemistry

Immunohistochemistry was performed on 3 μm formalin-fixed sections. Activin A and follistatin immunohistochemistry were

performed as described.[6] For TGF-β and activin receptor immunostaining antigen retrieval was performed and endogenous peroxidise blocked in H_2O_2. Sections were stained with antibodies specific for TGF-β1 (sc-146; Santa Cruz Biotechnology, Inc. Santa Cruz, California, USA), or ActRIB/ALK4, ActRIIA and ActRIIB (N-20, sc-11984; N-17, sc-5667; and N-16, sc-5665, respectively; Santa Cruz Biotechnology, Inc.) or appropriate isotype controls. See online supplement.

Quantitative image analysis

The frequency of periodic acid Schiff (PAS), activin A, follistatin or ActRIB positive airway epithelial cells per mm basement membrane was calculated (http://fiji.sc). Alternatively, airway epithelial TGF-β, activin A, ActRIIA and ActRIIB immunoreactivity was expressed as integrated density (n=5–19 airways/mouse). The percentage of subepithelial collagen in Masson's trichrome-stained sections was calculated to a depth of 20 μm below the basement membrane.[16] Smooth muscle thickness was measured at right angles across the muscle bundle on ActRIB-stained sections (n=2–7 airways/mouse). See online supplement.

Statistics

Data were analysed for normality and log transformed as necessary prior to analysis by analysis of variance and Tukey post tests or t tests (GraphPad Prism V.5.03). Differences were considered significant at $p<0.05$. Group sizes are indicated in the figure legends. All values in figures are mean±SEM.

RESULTS

Repeated allergen challenge induces acute resolving pulmonary inflammation

We investigated the effect of chronic allergen challenge on the kinetics of airway inflammation and remodelling to validate our model. Mice were challenged with 5, 25 or 100 μg OVA to investigate the effect of allergen dose (see online supplementary figure S1A). Regardless of OVA challenge dose, BAL cell counts were markedly increased compared with saline controls after 2 weeks of challenge, with eosinophils the major infiltrating cell type (figure 1A,B). After 5–10 weeks of challenge total BAL and eosinophil counts had decreased sharply but remained significantly higher than saline controls, and returned to baseline after challenges ceased. BAL lymphocytes followed a similar pattern (figure 1C). Cell counts in the lung-draining LN were elevated from 5 weeks and trended toward control values once allergen challenges stopped (12 weeks) (figure 1D). The frequency of IL-4-producing cells in the draining LN peaked after 2 weeks of challenge, decreasing gradually thereafter, but remaining above saline levels at 12 weeks (figure 1E). OVA-specific IgE levels were significantly elevated after 2 weeks of challenge, dropping sharply by 5 weeks and remaining stable thereafter (figure 1F). Thus allergen challenge induced acute inflammation at 2 weeks, similar to other acute asthma models, but this decreased sharply upon continued OVA challenge, and decreased further once allergen challenge ceased. Airway inflammation/eosinophilia was dose dependent, with the 100 μg group showing the greatest inflammation. The dose-dependent effect on serum OVA-specific IgE was reversed, with the 5 μg OVA challenge group having the highest level at all time points.

Allergen challenge increases BAL fluid levels of activin A, follistatin and TGF-β1

Next we determined the effect of chronic allergen challenge on concentrations of activin A, follistatin and TGF-β1 in the airways. Activin A and TGF-β1 concentrations were significantly elevated after 2 weeks of challenge, remaining high during allergen challenge, and dropping sharply once allergen challenge ceased (figure 2A,B). Follistatin concentrations showed a similar pattern, approaching control values by the 10-week time point (figure 2C). Thus, the elevated BAL fluid levels of activin A, TGF-β1 and follistatin seen during acute challenge are dependent on continued allergen challenge.

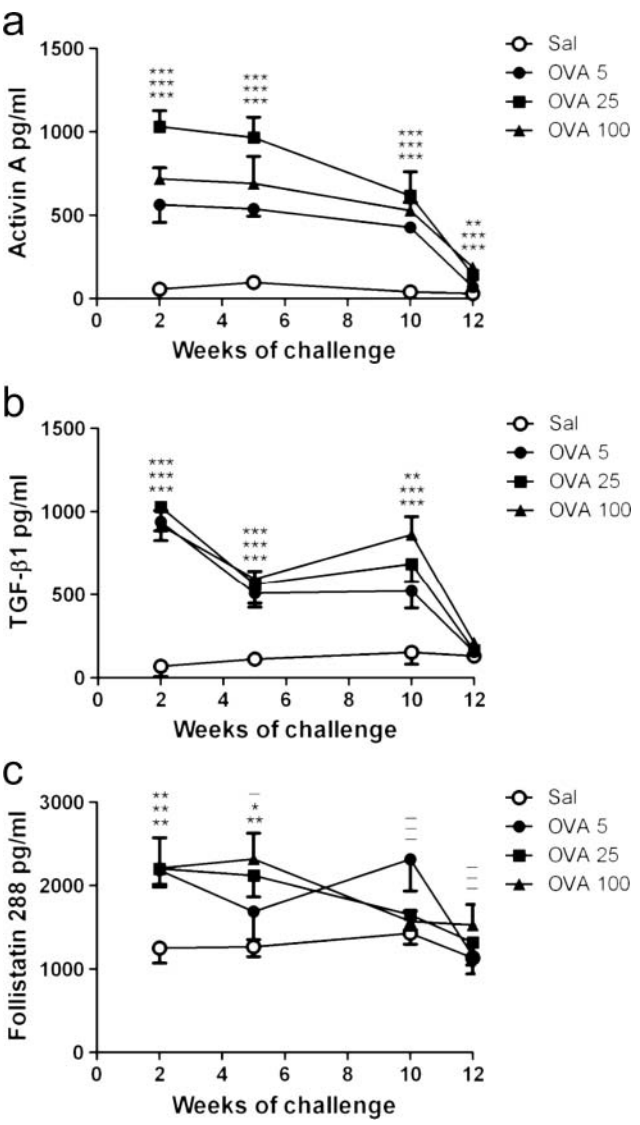


Figure 2 Activin A, TGF-β1 and follistatin levels are increased in bronchoalveolar lavage fluid during chronic allergen challenge. Mice were sensitised and challenged as described in online supplementary figure S1A, controls received saline instead of OVA. (A–C) Concentrations of activin A, TGF-β1 (ELISA) and follistatin (RIA) were measured at the time points shown in (A). Mean±SEM, n=6–7 mice/group per time point. *p<0.05, **p<0.01, ***p<0.001; the order of symbols from top to bottom is OVA 5, 25 and 100 μg (relative to saline).

Chronic allergen challenge decreases airway epithelial cell activin A and follistatin immunoreactivity, and induces airway remodelling

Airway epithelium in the saline group showed strong and uniform immunoreactivity for activin A and follistatin (figure 3A,C). In contrast, epithelial cell activin A and follistatin

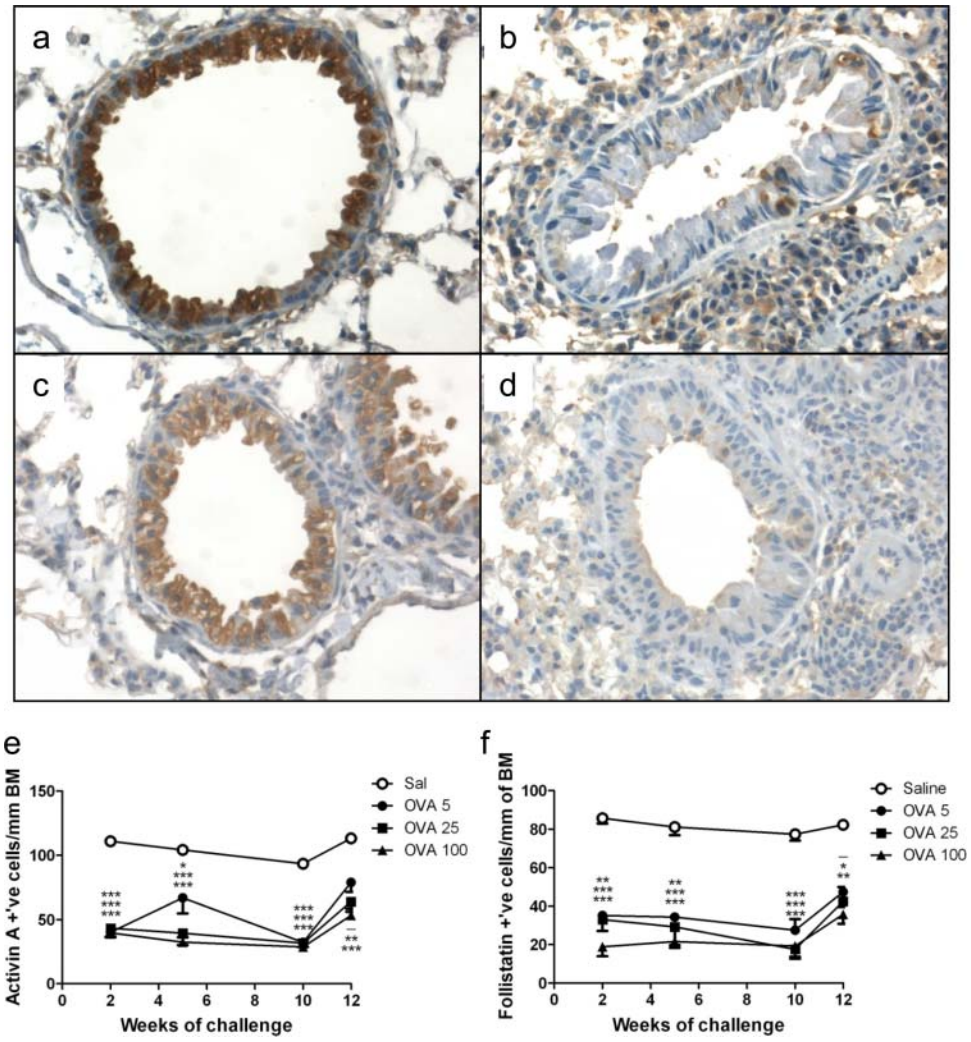


Figure 3 Activin A and follistatin expression by airway epithelium in the normal lung is lost during chronic allergen challenge. Mice were sensitised and challenged as described in online supplementary figure S1A, controls received saline instead of OVA. Representative lung tissue sections stained immunohistochemically for activin A (A, B) and follistatin (C, D) from saline control (A, C) and OVA sensitised and challenged mice (B, D). (E, F) Frequency of activin A and follistatin immunoreactive cells per millimetre basement membrane (BM) as determined by quantitative image analysis. (A–D) Original magnification ×400. (E, F) Mean±SEM, n=6–7 mice/group per time point. *p<0.05, **p<0.01, ***p<0.001, respectively; order of symbols from top to bottom is OVA 5, 25 and 100 μg.

immunoreactivity were dramatically decreased during chronic allergen challenge, remaining low throughout the challenge period, and sharply increasing once allergen challenge ceased (figure 3B,D,E,F). After 5 weeks of challenge there was a significant increase in subepithelial collagen, consistent with other chronic challenge models,[14–16] and this persisted at 10 weeks and after cessation of allergen challenge (figure 4A,B,E). The frequency of PAS-positive airway epithelial cells was significantly increased after 2 weeks of allergen challenge, remaining high throughout the challenge period, but declining sharply once allergen challenge stopped (figure 4C,D,F). Therefore, the increase in subepithelial collagen deposition persisted after cessation of allergen challenge, whereas mucus hypersecretion was dependent upon continued challenge. There was no consistent effect of allergen dose on the kinetics of these parameters.

Follistatin administration during chronic allergen challenge attenuates Th2 cytokine production

To investigate the role of activin A in the remodelling process we instilled follistatin at the time of allergen challenge. We previously showed that 0.5–1 μg FS288 instilled prior to allergen challenge inhibits allergen-specific T helper 2 (Th2) cytokine and airway epithelial mucus production in an acute asthma model.[6] Since activin A acts in a concentration-dependent manner[19] (our own unpublished data), we tested the effect of 0.05, 0.5 and 5 μg follistatin instilled during chronic allergen challenge (see online supplementary figure S1B). Given that remodelling was independent of the OVA challenge dose (figure 4), we used 25 μg OVA per challenge for the remainder of our studies. Compared with mice challenged with OVA alone, low-dose follistatin significantly increased the number of BAL eosinophils (figure 5A) and total BAL cells (not shown). Surprisingly, however, higher follistatin doses had no effect on BAL eosinophils or total BAL cell counts. Follistatin dose-dependently increased lung-draining LN cell counts (figure 5B), but decreased the frequency of IL-4, IL-5 and IL-13 producing cells in the lung-draining LN (figure 5C–E). Thus, overall there was a marginal decrease in numbers of Th2 cytokine-producing cells (see online supplementary figure S2). In contrast, BAL fluid IL-13 concentrations were only significantly decreased in mice treated with low-dose follistatin (see online

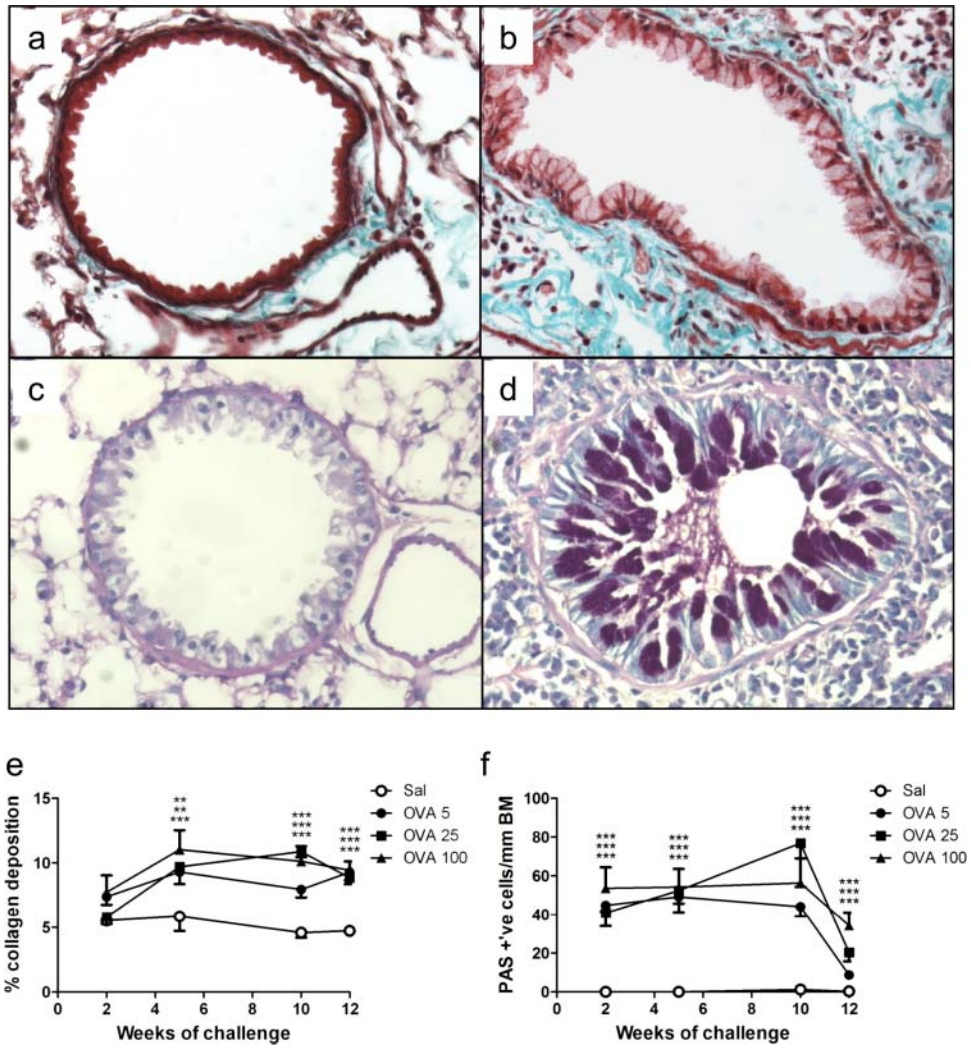


Figure 4 Chronic allergen challenge induces subepithelial collagen deposition and mucus hypersecretion. Mice were sensitised and challenged as described in online supplementary figure S1A, controls received saline instead of OVA. Representative lung tissue sections stained with Masson's Trichrome (A, B) or periodic acid Schiff (PAS) reagent (C, D) from saline control (A, C) and OVA sensitised and challenged mice (B, D). Collagen deposition in the subepithelial region (E) and frequency of PAS-positive airway epithelial cells per millimetre basement membrane (BM) (F) as determined by quantitative image analysis. (A–D) Original magnification ×400. (E, F) Mean±SEM, n=6–7 mice/group per time point. **p<0.01, ***p<0.001; order of symbols from top to bottom is OVA 5, 25 and 100 μg.

supplementary figure S3). We questioned whether the decreased frequency of Th2 cytokine-producing cells was mediated by regulatory T cells (Treg) or induction of a Th1-biased response. Lung $CD4^+CD25^+Foxp3^+$ Treg numbers and proportions were increased by chronic allergen challenge, as expected,[20] but were not further increased by follistatin treatment (see online supplementary figure S4). BAL fluid interferon γ levels were below the limit of detection in all treatment groups (data not shown). Follistatin instillation also dose-dependently decreased serum OVA-specific IgE levels (figure 5F). Thus follistatin exerts distinct dose-dependent effects in different pulmonary compartments, with a trend towards decreased frequency of Th2 cytokine-producing cells and serum OVA-specific IgE at the highest dose.

Follistatin during chronic allergen challenge inhibits BAL fluid activin A and TGF-β1 concentrations, and airway remodelling

Follistatin instillation during chronic allergen challenge dose-dependently decreased BAL fluid activin A concentrations, with the 5 μg follistatin group decreased to approximately saline levels (figure 6A). Similarly, high-dose follistatin significantly decreased BAL fluid TGF-β1 concentrations (figure 6B). Notably, quantitative image analysis demonstrated that follistatin at all three doses significantly decreased collagen deposition in the subepithelial region (figure 6C). Furthermore, 5 μg follistatin significantly decreased the frequency of airway epithelial mucus-producing cells (figure 6D). Thus, follistatin inhibited airway activin A and TGF-β1 levels, and mucus hypersecretion and subepithelial collagen deposition.

Follistatin inhibits the loss of airway epithelial TGF-β and ActRIB immunostaining during chronic allergen challenge

Immunohistochemistry showed strong TGF-β and ActRIB/ALK4 staining in normal (sal/sal) airway epithelium, which was significantly decreased in chronically challenged mice, and this decrease was significantly inhibited by follistatin (figures 7 and 8). There was strong immunostaining for ActRIB in the subepithelial smooth muscle of large airways in all groups. Subepithelial smooth muscle thickness was significantly increased more than twofold in OVA/OVA mice, and there was a

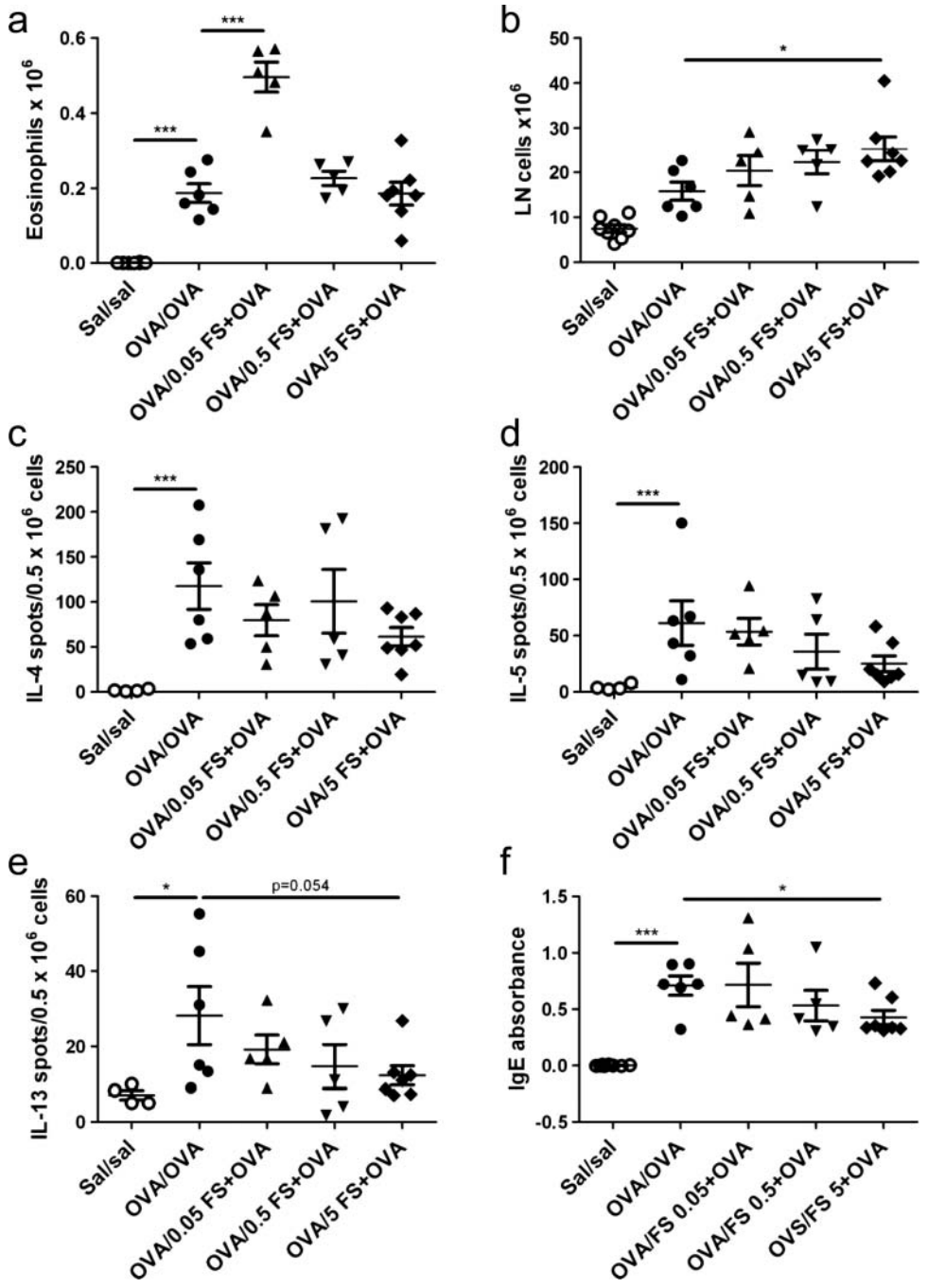


Figure 5 High-dose follistatin instillation during chronic allergen challenge inhibits allergen-specific T helper 2 cytokine production in the lung draining LN and serum allergen-specific IgE. Mice were sensitised as described in online supplementary figure S1B, and challenged with OVA, or OVA and follistatin; controls received saline instead of OVA or follistatin. Number of bronchoalveolar lavage eosinophils (A) and lung-draining LN cells (B). (C–E) Frequency of OVA-specific interleukin (IL)-4, IL-5 and IL-13 producing cells in the lung-draining LN. (F) Levels of serum OVA-specific IgE. Mean±SEM, n=6–8 mice/group. *p<0.05, ***p<0.001. FS, follistatin; Sal, saline.

strong trend for inhibition of this increase by 5 µg follistatin (p=0.09) (figure 8A,C). The strong immunoreactivity for activin A, ActRIIA and ActRIIB in normal airway epithelium mice was significantly decreased in chronically challenged mice, but this was not affected by follistatin treatment (see online supplementary figures S5 and S6). Apart from occasional activin A-positive macrophages in the lamina propria, there was weak and/or diffuse activin A, TGF-β and activin receptor staining of inflammatory cells in the OVA/OVA and OVA/5 FS+OVA groups.

DISCUSSION

In most patients stable asthma is well controlled on current medication but management of severe asthma remains inadequate. Despite the importance of inflammation in asthma pathogenesis, drugs targeting key inflammatory cells and inflammatory mediators show limited clinical benefit.[1] These disappointing results suggested a reappraisal of asthma pathogenesis.[1] Accumulating evidence indicates that activin A drives tissue fibrosis in a variety of organs,[21–24] and is implicated in airway remodelling.[12 13] In the current study we established a murine

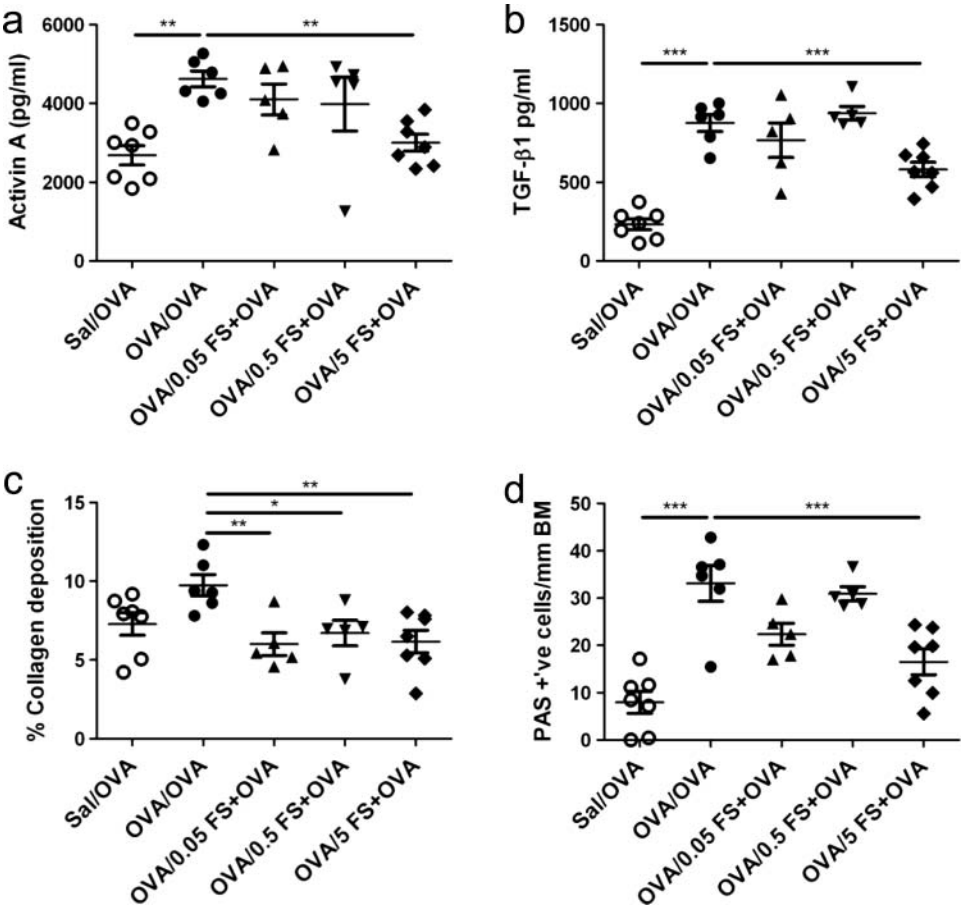


Figure 6 Follistatin instillation during chronic allergen challenge inhibits bronchoalveolar lavage (BAL) fluid levels of activin A and TGF-β1, subepithelial collagen deposition and mucus hypersecretion. Mice were sensitised as described in online supplementary figure S1B, and challenged with OVA, or OVA and follistatin; controls received saline instead of OVA or follistatin. BAL fluid concentrations of activin A (A) and TGF- β1 (B). (C) Collagen deposition in the subepithelial region (D) and frequency of periodic acid Schiff (PAS)-positive airway epithelial cells per millimetre basement membrane as determined by quantitative image analysis. Mean±SEM, n=6–8 mice/group. *p<0.05, **p<0.01, ***p<0.001. FS, follistatin; Sal, saline.

model of chronic allergic asthma to investigate the ability of follistatin to inhibit development of airway remodelling. Our results show that high-dose follistatin decreased airway activin A and TGF-β1 concentrations, and inhibited the decrease in TGF-β and ActRIB/ALK4 immunostaining observed in the airway epithelium of chronically challenged mice. Follistatin further inhibited mucus hypersecretion, subepithelial collagen deposition and thickening of the subepithelial smooth muscle, indicating a potential therapeutic role for follistatin in the prevention of airway remodelling.

Our data show that BAL fluid concentrations of activin A are elevated after 2 weeks of challenge, the earliest time point examined. Our previous studies show that increases in BAL fluid activin A concentrations occur within 24 h after allergen challenge,[6] consistent with reports of increased activin A after one to three allergen challenges.[10 13] The increased BAL fluid activin A and loss of activin A immunolocalisation in airway epithelium were rapidly reversed upon cessation of allergen challenge. These changes in airway and epithelial activin A expression mirrored the pattern seen for TGF-β1, consistent with other reports,[14 25] suggesting release of activin A from epithelium into the airspaces. Similar changes were observed for follistatin, supporting the idea that follistatin is rapidly released as an endogenous antagonist of activin A bioactivity.[6 26] One factor driving altered activin A compartmentalisation is IL-13,[9] a key instigator of airway remodelling.[15 27 28] Our observation that BAL fluid concentrations of TGF-β1 showed almost identical kinetics to activin A is not surprising since activin A and TGF-β1 stimulate secretion of one another,[5 29–31] and IL-13 stimulates production of activin A and TGF-β1.[9 27]

Follistatin instillation during chronic allergen challenge decreased BAL fluid activin A and TGF-β1 concentrations. Since follistatin inhibited the loss of airway epithelial TGF-β during chronic challenge, the decreased BAL fluid TGF-β could simply reflect its decreased liberation from airway epithelium. The decreased BAL fluid activin A levels are likely due to formation of follistatin–activin A complexes. Subsequently, the decrease in available activin A would lead to decreased BAL fluid TGF-β1.[5 29–31] Given the key role for TGF-β1 in airway remodelling,[27] a simplistic interpretation would be that follistatin attenuated airway remodelling via inhibition of TGF-β1. However, follistatin binds to activin A with high affinity, but does not bind TGF-β1 or TGF-β2.[32 33] Furthermore, activin A has been implicated in lung fibrosis via promotion of collagen and α-smooth muscle actin synthesis, and proliferation of human airway fibroblasts and smooth muscle,[5 10 11] and follistatin decreases collagen secretion by pancreatic and hepatic stellate cells, and renal fibroblasts.[29–31] Receptor-blocking experiments using dominant negative activin A and TGF-β type II receptors suggested that TGF-β action is partly mediated by secreted activin A.[30] Together, these studies suggest that follistatin inhibits airway remodelling by blocking activin A, thereby inhibiting TGF-β1 production, positioning activin A as a central regulator of fibrosis.

We observed a general loss of activin receptor staining in airway epithelium following chronic allergen challenge. The thickened subepithelial smooth muscle showed strong ActRIB/ALK4 immunostaining but weak immunostaining for ActRIIA and ActRIIB. Subepithelial cells in the same location and with similar morphology stain with α-smooth muscle actin,[16] emphasising that the ActRIB/ALK4-stained cells are smooth muscle or

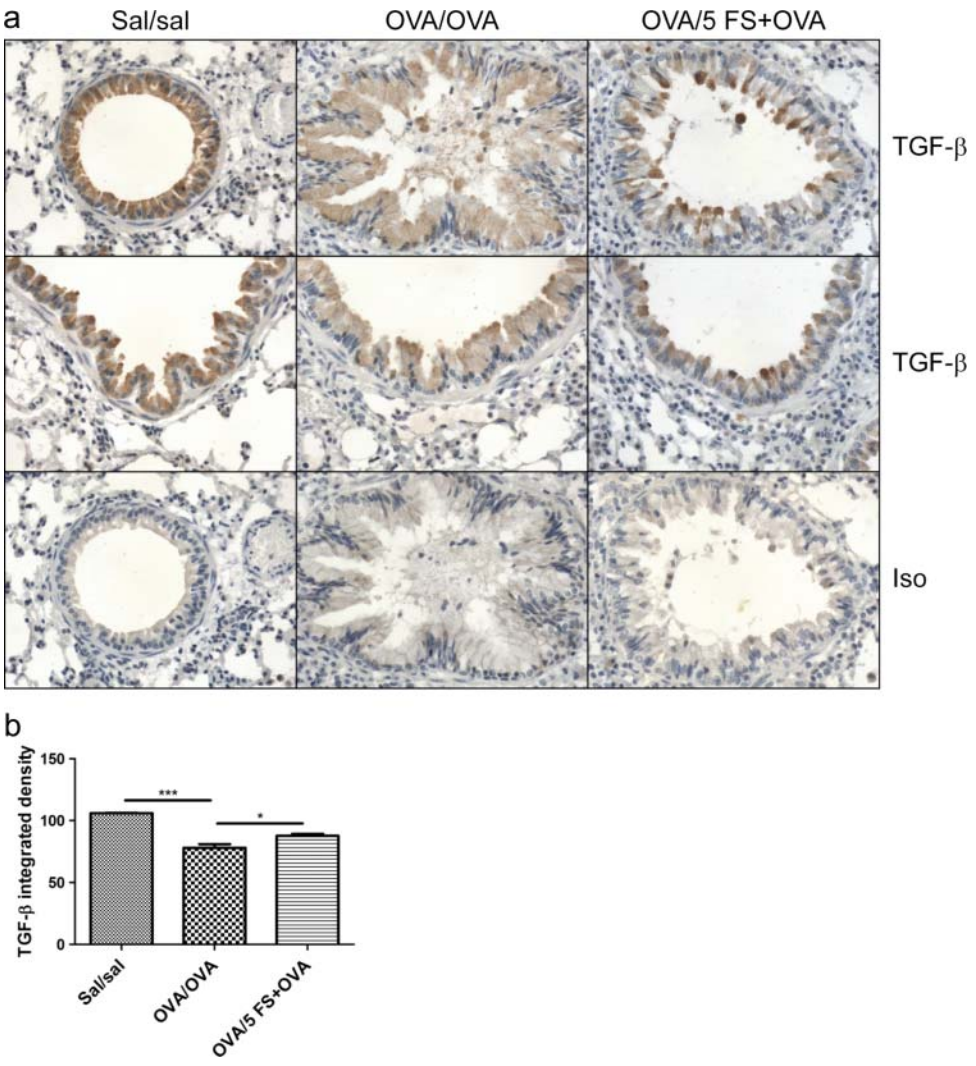


Figure 7 Follistatin treatment during chronic allergen challenge diminishes the loss of TGF-β immunoreactivity in airway epithelium. Mice were sensitised as described in online supplementary figure S1B, and challenged with OVA, or OVA and follistatin; controls received saline instead of OVA or follistatin. (A) Formalin-fixed lung sections were stained immunohistochemically with antibody to TGF-β1. Representative micrographs, original magnification ×400. (B) Airway epithelial TGF-β staining intensity in small–medium airways. Mean±SEM, n=3 mice/group. *p<0.05, ***p<0.001. FS, follistatin; Sal, saline.

myofibroblasts. Similarly, decreased activin type I and II receptor expression was observed in airway epithelium and/or subepithelial cells in patients with asthma.[7] In contrast, increased ALK4 and ActRIIA expression was found in airway epithelium of OVA-sensitised mice following acute allergen challenge,[34] and increased ALK4 and ActRIIA expression was found in asthmatic airway epithelium 24 h after allergen challenge.[8] Conceivably, differences in activin receptor expression between studies are due to different kinetics, with increased activin receptor expression occurring early after allergen challenge, decreasing at later times (ie, 5 weeks).

Chronic inflammation may be a driver of airway remodelling.[1] However, several studies report dissociation between inflammation and airway remodelling. Neutralisation of TGF-β1 specifically, or all TGF-β isoforms, in OVA chronic allergen challenge models suppressed pulmonary fibrosis, without affecting airway inflammation, eosinophilia, or IL-5 or IL-13 production.[25 35] Furthermore, overexpression of the activin/TGF-β1 signalling intermediate Smad2 in airway epithelium exacerbated airway remodelling in a house dust mite allergen challenge model.[13] Intraperitoneal injection of activin A neutralising antibody markedly inhibited airway remodelling, but had no effect on lung inflammation, airway eosinophilia or Th2 cytokine production. Our data demonstrate that while follistatin dose-dependently modulated inflammation, the inhibition of subepithelial fibrosis was dose independent. However, BAL fluid activin A and TGF-β1 levels were only inhibited by high-dose follistatin, suggesting that even subtle decreases in local tissue concentrations of activin A and TGF-β1 can decrease fibrosis. Thus, while ELISA for activin A and TGF-β1 in the BAL fluid provides insight into lung levels of these mediators, it does not exactly mirror changes in mediator concentrations in the local tissue microenvironment.

Recently, antibody-mediated neutralisation of activin A during allergen challenge was demonstrated to exacerbate acute allergic airway inflammation.[7] However, in our model of chronic allergic inflammation, inhibiting activin A with intermediate or high-dose intranasal follistatin suppressed OVA-specific IgE and allergen-specific Th2 cytokine production, and had no effect on airway eosinophilia. The attenuated Th2 cytokine production in the draining LN was not due to follistatin-induced expansion of $CD4^+CD25^+Foxp3^+$ Treg in the lung, nor was it due to a

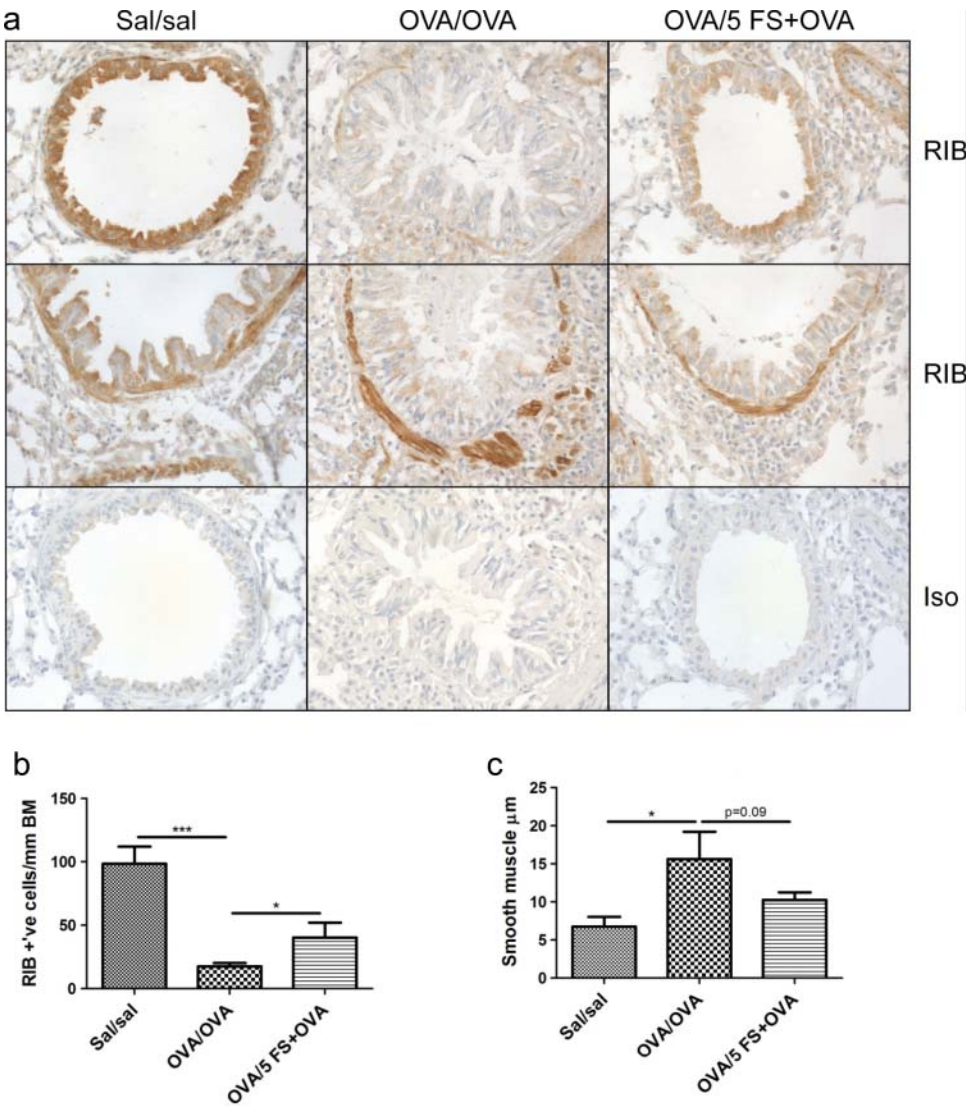


Figure 8 Follistatin treatment during chronic allergen challenge diminishes the loss of ActRIB/ALK4 immunoreactivity in airway epithelium. Mice were sensitised as described in online supplementary figure S1B, and challenged with OVA, or OVA and follistatin; controls received saline instead of OVA or follistatin. (A) Formalin-fixed lung sections were stained immunohistochemically with antibody to ActRIB. Representative micrographs, original magnification ×400. Frequency of ActRIB-positive airway epithelial cells in small–medium airways (B) and subepithelial muscle thickness in large airways (C). Mean±SEM, n=3 mice/group. *p<0.05, ***p<0.001. FS, follistatin; Sal, saline.

switch to a Th1-type immune response. Thus, follistatin must exert this dampening effect via the induction of an alternative suppressive mechanism, such as the selective deletion/apoptosis of Th2 cells. These data suggest that the outcome of blocking activin A depends on the method (antibody vs follistatin) and/or the site (systemic vs local) of inhibition. Our finding that the lowest follistatin dose (0.05 µg) enhanced airway eosinophilia points to concentration-dependent effects of activin A on immune function. Analogous concentration-dependent morphogen effects of activin A occur during development.[19]

In summary, our study shows that follistatin inhibited activin A and TGF-β1 secretion into the airway lumen during chronic allergen challenge, and significantly decreased subepithelial fibrosis and airway epithelial mucus production. Follistatin treatment also caused a dose-dependent modulation of allergic airway inflammation, suggesting 'morphogen-like' effects of activin A on immune function. This is consistent with apparently contradictory observations of pro-inflammatory versus anti-inflammatory effects of activin A in other systems.[2] Our study reinforces the idea that activin A is a key driver of inflammation and fibrosis, and indicates that follistatin represents an attractive potential therapeutic for the prevention of fibrosis in asthma.

Acknowledgements The authors thank Professor Gary Anderson (University of Melbourne) for advice on establishing the mouse chronic asthma model and Ms Genevieve Tan (Department of Immunology, Monash University) for assistance with image analysis. We are grateful to Professor Jennifer Wilkinson-Berka and Pam Tong Zhu (Department of Immunology, Monash University) for advice with the TGF-β immunohistochemistry, and Elizabeth Richards (Department of Biochemistry and Molecular Biology, Monash University) for assistance with the activin A and activin receptor immunohistochemistry.

Contributors CLH wrote the paper, designed, performed and analysed experiments. HN designed, performed and analysed experiments, and produced rhFS288. RM performed and analysed experiments. JY performed experiments. DO analysed experiments. MP provided intellectual guidance. KL provided advice and helped with techniques. CH helped produce rhFS288. JM wrote the paper and provided intellectual guidance. ROH wrote the paper and provided intellectual guidance.

Funding Supported by grants from the National Health and Medical Research Council and the Co-operative Research Centre for Asthma and Airways, Australia.

Competing interests None.

Ethics approval This study was conducted with the approval of the Alfred Medical Research and Education Precinct Animal Ethics Committee.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1 Holgate ST. Asthma: a simple concept but in reality a complex disease. *Eur J Clin Invest* 2011;41:1339–52.
2 Hedger MP, Winnall WR, Phillips DJ, *et al*. The regulation and functions of activin and follistatin in inflammation and immunity. *Vitam Horm* 2011;85:255–97.
3 de Kretser DM, O'Hehir RE, Hardy CL, *et al*. The roles of activin A and its binding protein, follistatin, in inflammation and tissue repair. *Mol Cell Endocrinol* 2012;359:101–6.
4 Phillips DJ. Regulation of activin's access to the cell: why is mother nature such a control freak? *Bioessays* 2000;22:689–96.
5 Karagiannidis C, Hense G, Martin C, *et al*. Activin A is an acute allergen-responsive cytokine and provides a link to TGF-beta-mediated airway remodeling in asthma. *J Allergy Clin Immunol* 2006;117:111–18.
6 Hardy CL, O'Connor AE, Yao J, *et al*. Follistatin is a candidate endogenous negative regulator of activin A in experimental allergic asthma. *Clin Exp Allergy* 2006;36:941–50.
7 Semitekolou M, Alissafi T, Aggelakopoulou M, *et al*. Activin-A induces regulatory T cells that suppress T helper cell immune responses and protect from allergic airway disease. *J Exp Med* 2009;206:1769–85.
8 Kariyawasam HH, Pegorier S, Barkans J, *et al*. Activin and transforming growth factor-beta signaling pathways are activated after allergen challenge in mild asthma. *J Allergy Clin Immunol* 2009;124:454–62.
9 Hardy CL, Lemasurier JS, Olsson F, *et al*. Interleukin-13 regulates secretion of the tumor growth factor-{beta} superfamily cytokine activin A in allergic airway inflammation. *Am J Respir Cell Mol Biol* 2010;42:667–75.
10 Cho SH, Yao Z, Wang SW, *et al*. Regulation of activin A expression in mast cells and asthma: its effect on the proliferation of human airway smooth muscle cells. *J Immunol* 2003;170:4045–52.
11 Aoki F, Kurabayashi M, Hasegawa Y, *et al*. Attenuation of bleomycin-induced pulmonary fibrosis by follistatin. *Am J Respir Crit Care Med* 2005;172:713–20.
12 Le AV, Cho JY, Miller M, *et al*. Inhibition of allergen-induced airway remodeling in Smad 3-deficient mice. *J Immunol* 2007;178:7310–16.
13 Gregory LG, Mathie SA, Walker SA, *et al*. Overexpression of Smad2 drives house dust mite-mediated airway remodeling and airway hyperresponsiveness via activin and IL-25. *Am J Respir Crit Care Med* 2010;182:143–54.
14 McMillan SJ, Lloyd CM. Prolonged allergen challenge in mice leads to persistent airway remodelling. *Clin Exp Allergy* 2004;34:497–507.
15 Kumar RK, Herbert C, Yang M, *et al*. Role of interleukin-13 in eosinophil accumulation and airway remodelling in a mouse model of chronic asthma. *Clin Exp Allergy* 2002;32:1104–11.
16 Leigh R, Ellis R, Wattie J, *et al*. Dysfunction and remodeling of the mouse airway persist after resolution of acute allergen-induced airway inflammation. *Am J Respir Cell Mol Biol* 2002;27:526–35.
17 Hardy CL, Kenins L, Drew AC, *et al*. Characterization of a mouse model of allergy to a major occupational latex glove allergen Hev b 5. *Am J Respir Crit Care Med* 2003;167:1393–9.
18 O'Connor AE, McFarlane JR, Hayward S, *et al*. Serum activin A and follistatin concentrations during human pregnancy: a cross-sectional and longitudinal study. *Hum Reprod* 1999;14:827–32.
19 Gurdon JB, Dyson S, St Johnston D. Cells' perception of position in a concentration gradient. *Cell* 1998;95:159–62.
20 Strickland DH, Stumbles PA, Zosky GR, *et al*. Reversal of airway hyperresponsiveness by induction of airway mucosal CD4+CD25+ regulatory T cells. *J Exp Med* 2006;203:2649–60.
21 Gaedeke J, Boehler T, Budde K, *et al*. Glomerular activin A overexpression is linked to fibrosis in anti-Thy1 glomerulonephritis. *Nephrol Dial Transplant* 2005;20:319–28.
22 Patella S, Phillips DJ, Tchongue J, *et al*. Follistatin attenuates early liver fibrosis: effects on hepatic stellate cell activation and hepatocyte apoptosis. *Am J Physiol Gastrointest Liver Physiol* 2006;290:G137–44.
23 Yndestad A, Ueland T, Oie E, *et al*. Elevated levels of activin A in heart failure: potential role in myocardial remodeling. *Circulation* 2004;109:1379–85.
24 Munz B, Smola H, Engelhardt F, *et al*. Overexpression of activin A in the skin of transgenic mice reveals new activities of activin in epidermal morphogenesis, dermal fibrosis and wound repair. *EMBO J* 1999;18:5205–15.
25 Alcorn JF, Rinaldi LM, Jaffe EF, *et al*. Transforming growth factor-beta1 suppresses airway hyperresponsiveness in allergic airway disease. *Am J Respir Crit Care Med* 2007;176:974–82.
26 Jones KL, De Kretser DM, Clarke IJ, *et al*. Characterisation of the rapid release of activin A following acute lipopolysaccharide challenge in the ewe. *J Endocrinol* 2004;182:69–80.
27 Lee CG, Homer RJ, Zhu Z, *et al*. Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor beta(1). *J Exp Med* 2001;194:809–21.
28 Zhu Z, Homer RJ, Wang Z, *et al*. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest* 1999;103:779–88.
29 Ohnishi N, Miyata T, Ohnishi H, *et al*. Activin A is an autocrine activator of rat pancreatic stellate cells: potential therapeutic role of follistatin for pancreatic fibrosis. *Gut* 2003;52:1487–93.
30 Wada W, Kuwano H, Hasegawa Y, *et al*. The dependence of transforming growth factor-beta-induced collagen production on autocrine factor activin A in hepatic stellate cells. *Endocrinology* 2004;145:2753–9.
31 Yamashita S, Maeshima A, Kojima I, *et al*. Activin A is a potent activator of renal interstitial fibroblasts. *J Am Soc Nephrol* 2004;15:91–101.
32 Iemura S, Yamamoto TS, Takagi C, *et al*. Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early Xenopus embryo. *Proc Natl Acad Sci U S A* 1998;95:9337–42.
33 Harrington AE, Morris-Triggs SA, Ruotolo BT, *et al*. Structural basis for the inhibition of activin signalling by follistatin. *EMBO J* 2006;25:1035–45.
34 Rosendahl A, Checchin D, Fehniger TE, *et al*. Activation of the TGF-beta/activin-Smad2 pathway during allergic airway inflammation. *Am J Respir Cell Mol Biol* 2001;25:60–8.
35 McMillan SJ, Xanthou G, Lloyd CM. Manipulation of allergen-induced airway remodeling by treatment with anti-TGF-beta antibody: effect on the Smad signaling pathway. *J Immunol* 2005;174:5774–80.

Supplemental information, Hardy et al.

Recombinant follistatin

Follistatin 288 (FS288) was produced using the follistatin expressing plasmid (pSV2HF288), a gift from Professor Shunichi Shimasaki (University of California, San Diego, USA). The FS288 gene was amplified from pSV2HF288 by PCR and sub-cloned into pAPEX3P vector. The pAPEX3P-FS288 plasmid was transfected into 293EBNA cells, and puromycin-resistant cells expanded to form the stable 293EBNA FS cell line. FS288 was purified from conditioned media of cultured 293EBNA FS cells by successive rounds of chromatography through heparin-Sepharose affinity (5 ml Hi-Trap Heparin column, GE Healthcare Bio-Sciences), size exclusion (Superdex 200 prep grade, Hi-load 16/60) and RP-HPLC (Reversed Phase, OD-300, Aquapore ODS, C-18, 7 um, 300 A, 10 cm, 2.1 mm i.d. Brownlee Cartridge Column; PerkinElmer) columns.

Follistatin luciferase bioassay

To assess the ability of recombinant follistatin to inhibit activin activity, HEK293T cells, plated on poly-lysine-coated 24-well plates at a density of 150,000 cells per well, were co-transfected with 50 ng A3-luc (a Smad2-responsive luciferase reporter), 25 ng FAST-2 (a transcriptional co-activator), and 25 ng β-galactosidase (to normalise for transfection efficiency). Transfections were performed under optimized conditions using lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). 16 h post-transfection, cells were treated with activin-A and increasing doses of recombinant human FS288 (rhFS288) for ~16 h. Cells were harvested in solubilization buffer (1% Triton X-100, 25 mM glycylglycine (pH 7.8), 15 mM MgSO4, 4 mM EGTA, and 1 mM dithiothreitol), and luciferase reporter activity was measured (Victor2 Multilabel Counter; Perkin Elmer, Waltham, MA) and normalized relative to β-galactosidase activity.

Bronchoalveolar lavage, differential counts, and tissue sampling

Methods were as described previously [1]. Blood was collected from the inferior vena cava, and serum separated by centrifugation. Bronchoalveolar lavage (BAL) was performed

with 0.4 ml 1% FCS in PBS followed by three lavages of 0.3 ml. Viable lung-draining lymph node (LN) cells and BAL leukocytes were counted in a hemocytometer. For differential cell counts, BAL cytospots were stained with Giemsa (Merck, Kilsyth, Victoria, Australia), and 200 cells were identified by morphologic criteria. Lungs were formalin fixed prior to paraffin embedding.

Lung tissue digests

Tissue digestion was performed as described [2] with modifications. The right ventricle was perfused with 5 ml Ca^{2+}/Mg^{2+}-free HBSS (Invitrogen #14175095) with 0.01 M EDTA, pH 7.2. Lung and draining LN were chopped with a tissue chopper (Mickle Laboratory Engineering Co. Ltd, Gomshall, Surrey, UK). Tissue fragments were digested in collagenase type III (1 mg/ml; Worthington, Lakewood, NJ, USA) and DNase type I (0.025 mg/ml; Roche Diagnostics, Sydney NSW #1284932) in a volume of 7 ml at 25°C by manual pipetting for 20 minutes. The reaction was stopped by adding one 10^{th} volume of 0.01 M EDTA and mixing for 5 minutes. The cell suspension was filtered through a 70 μm cell strainer (BD Falcon) and underlaid with 1 ml 0.01 M EDTA in FCS prior to centrifugation (350 *g*, 4°C). The cell pellet was resuspended in red cell lysis solution for 3–5 min (#R7757, Sigma-Aldrich, St. Louis, MO, USA), diluted to 10 ml in RPMI and 10% FCS, and underlaid with 1 ml 0.01 M EDTA in FCS prior to centrifugation (350 *g*, 4°C). Cells were resuspended in staining buffer [3% FCS, 3% pooled normal mouse serum, 5 mM EDTA (pH 7.2) and 0.1% Na-Azide in Ca^{2+}/Mg^{2+}-free HBSS], and viable cells counted in a haemocytometer.

Flow cytometry

Non-specific FcR binding was blocked by incubating cells in CD16/CD32 block (BD Biosciences, San Jose, CA, USA). Cells (1×10^6) were surface stained on ice for 20 minutes with the following antibodies: CD3-PE, CD4-V450, CD25-FITC (all from BD Biosciences). Cells were fixed and permeabilized (eBioscience, Inc., San Diego, CA, USA, # 00-5521-00) according to the manufacturer's instructions, prior to intracellular staining with Foxp3-APC (eBioscience). Appropriate isotype control antibodies were used. All dilutions were in

staining buffer (see above). Data was acquired on a LSR II (BD) and analysed on FlowJo (Tree Star, Ashland, OR, USA).

Cytokine ELISPOT

IL-4, IL-5, and IL-13 ELISPOT were performed as described previously [3]. Briefly, ELISPOT plates (Millipore, North Ryde, NSW, Australia) were coated with IL-4, IL-5 (BD Pharmingen, San Jose, CA), or IL-13 antibody (R&D Systems, Minneapolis, MN) and blocked with 10% FCS in RPMI 1640 (Invitrogen, Mt. Waverly, Victoria, Australia). LN cells (0.5×10^6) resuspended in 100 µl RPMI/10% FCS were added to the wells with 10 µl of OVA (final concentration, 25 µg/ml) or RPMI/10% FCS alone; triplicate cultures were used throughout. Plates were incubated for 16 hours at 37°C. Cytokine detection was with biotinylated IL-4, IL-5 (BD Pharmingen), or IL-13 antibodies (R&D Systems) followed by ExtrAvidin–alkaline phosphatase (E-2636; Sigma-Aldrich). Reaction product was developed (170-6432; Bio-Rad, Regents Park, NSW, Australia), and plates were read on an ELISPOT reader (Autoimmun Diagnostika, Strassberg, Germany).

OVA-specific IgE ELISA

OVA-specific IgE was detected as described previously [1]. Briefly, ELISA plates were coated with OVA (10 µg/ml), blocked with 5% skim milk powder/0.05% Tween 20 in PBS for 1.5 hours, and incubated with IgG-depleted serum diluted 1:5 followed by anti-mouse IgE-biotin and streptavidin-peroxidase. Absorbance was read at 490 nm; results are expressed as raw optical density readings minus background (no serum added).

Activin A ELISA

Activin A concentrations were measured using a specific ELISA according to the manufacturer's instructions (Oxford Bio-Innovations, Upper Heyford, Oxfordshire, UK) with modifications as described previously [3-4]. The activin A antibody (E4) was obtained from Professor Nigel Groome (Oxford Brookes University, UK). Note that 1 nM activin A = 25 ng/ml. The limit of activin A detection for serum and BAL fluid samples was 0.01 ng/ml. In some experiments, activin A was measured using a different commercial ELISA kit

according to the manufacturer's instructions (#DY338, R&D Systems). We observe a good correlation between values obtained with these two ELISA systems (data not shown).

Follistatin radioimmunoassay

Follistatin concentrations were measured using a discontinuous radioimmunoassay as described previously [4]. The follistatin rabbit antiserum (#204) used for the immunoassay was developed in house against purified native bovine follistatin. Human recombinant follistatin was used as the standard, and [^{125}I]-rhFS288 was used as the tracer. The assay buffer for the BAL fluid samples was 0.05% BSA/PBS. Note that 1 nM follistatin = 35–45 ng/ml (range due to variable glycosylation and different isoforms). The limit of follistatin detection was 1.04 ng/ml.

TGF-β1 ELISA

TGF-β1 was detected using a TGF-β1 ELISA kit according to the manufacturer's instructions (#DY1679; R&D Systems). Briefly, Costar ELISA plates (Corning Inc., Corning, NY) were coated with TGF-β1 antibody and blocked (5% Tween 20 in PBS with 0.05% NaN_3). Latent TGF-β1 was acid activated with 1 N HCl and neutralized with 1.2 N NaOH/0.5 M HEPES, and samples were adjusted to pH 7.2–7.6. Activated samples and serially diluted TGF-β1 standards were added and incubated for 2 hours. After washing, biotinylated TGF-β1–specific antibody was added for 2 hours. Detection was with streptavidin-horseradish peroxidase, and development was with *o*-phenylenediamine (P5412; Sigma-Aldrich). Absorbance was read at 490 nm. The limit of TGF-β1 detection was 8 pg/ml.

Immunohistochemistry

Paraffin sections were dewaxed and rehydrated, and antigens retrieved by immersing slides in 0.01 M citrate buffer (pH 6.0), heating in a 1,000 W microwave oven (high for 2.5 min, low for 5 min), cooling at 4°C for 20 min, and washing in water for 5 min. Endogenous peroxidase was blocked in 3% H_2O_2 for 10 min, and nonspecific binding was blocked for 1 hour (CAS-block [00-8120; Zymed Laboratories, South San Francisco, CA] and 10% normal

rabbit serum). Sections were incubated with mouse monoclonal antibodies specific for the activin βA subunit (E4; $IgG2_b$) (obtained from Professor Nigel Groome) or follistatin (2E6; IgM) at 10 μg/ml overnight at 4℃. After washing, slides were incubated in rabbit anti-mouse IgG_{2b} peroxidase (61-0320; Zymed) or -IgM peroxidase (61-6820; Zymed) diluted 1:500 for 2 hours for activin A and follistatin primary antibodies, respectively. Slides were washed in Tris-buffered saline (TBS), 0.05% Tween-20 (pH 7.5) and then MilliQ H_2O. Reaction product was developed with a 3,3′-diaminobenzidine tetrahydrochloride substrate kit (00-2014; Zymed), and sections were counterstained in Harris' hematoxylin. All wash steps were in TBS/0.05% Tween-20. Antibodies were diluted in 1% BSA/TBS. Nonimmune mouse antibodies of the appropriate immunoglobulin isotype were used for negative controls (02-6800 and 02-6300 for IgM and IgG_{2b}, respectively; Zymed). For TGF-β immunostaining antigens were retrieved in 0.01 M citrate buffer, and endogenous peroxidise blocked (3% H_20_2) prior to staining with TGF-β1−specific antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, CA U.S.A., sc-146), or rabbit IgG isotype control (sc-2027) using the rabbit ABC staining system (sc-2018). For activin receptor staining antigens were retrieved in 50 mM glycine pH 3.5, and endogenous peroxidise blocked (3% H_20_2) prior to staining with antibodies specific for ActRIB/ALK4 (Santa Cruz, N-20, sc-11984), ActRIIA (Santa Cruz, N-17, sc-5667) and ActRIIB (Santa Cruz, N-16, sc-5665) or goat IgG isotype control.

Image analysis

For analysis of PAS, activin A, follistatin and ActRIB staining, images of lung airways (basement membrane circumference 400-700 μm) were captured using a 40 x objective, and the number of positive cells per mm linear basement membrane calculated using appropriately calibrated Fiji Open Source image analysis software (http://fiji.sc). For TGF-β, activin A, ActRIIA and ActRIIB staining integrated pixel density of peroxidase-positive cells was calculated (http://fiji.sc); this type of analysis gave an almost identical pattern of results to that obtained with the frequency analysis. Thickness of the subepithelial smooth muscle in large airways was measured at right angles across the muscle bundle, at 8 equidistant points around the airway (images captured with the 20 x objective: http://fiji.sc).

Figure legends

Figure S1. Schedule of allergen immunisations and follistatin treatments for chronic allergen challenge models. Mice were sensitised i.p. with OVA/alum, while controls received sal/alum. (a) For the chronic time-course experiments mice were challenged 3 times/week with 5, 25 or 100 µg of OVA i.n., and groups of mice killed at the indicated time-points. (b) For the follistatin instillation studies mice were challenged with 25 µg OVA i.n. or OVA mixed with follistatin (0.05, 0.5 or 5 µg) for 5 wk.

Figure S2. High dose follistatin instillation during chronic allergen challenge causes a small decrease in total numbers of allergen-specific Th2 cytokine producing cells in the lung-draining LN. Mice were sensitised as described in Supplementary Figure 1b, and challenged with OVA, or OVA and follistatin; controls received saline instead of OVA or follistatin. (a–c) Absolute numbers of OVA-specific IL-4, IL-5 and IL-13 producing cells in the lung-draining LN. Mean ± sem, n=6–8 mice/group. **, ***; $p < 0.01$, 0.001, respectively.

Figure S3. Follistatin instillation during chronic allergen challenge inhibits BAL fluid levels of IL-13. Mice were sensitised as described in Supplementary Figure 1b, and challenged with OVA, or OVA and follistatin; controls received saline instead of OVA or follistatin. BAL fluid concentrations of IL-13. Mean ± sem, n=5–7 mice/group. *, **; $p < 0.05$, 0.01, respectively.

Figure S4. Chronic allergen challenge increases numbers of $CD4^+CD25^+Foxp3^+$ Treg in the lung, and this is not affected by follistatin. Mice were sensitised as described in Supplementary Figure 1b, and challenged with OVA, or OVA and follistatin; controls received saline instead of OVA or follistatin. Lung leukocytes were isolated by collagenase/DNase digestion, and gated on viable $CD3^+$ cells. Data shows proportions (a) and numbers (b) of $CD4^+CD25^+Foxp3^+$ cells. Mean ± sem, n=3–4 replicates/group (pools of 2–3 mice per replicate). ***; $p < 0.001$.

Figure S5. Chronic allergen challenge causes loss of activin A immunoreactivity in airway epithelium. Mice were sensitised as described in Supplementary Figure 1b, and challenged with OVA, or OVA and follistatin; controls received saline instead of OVA or follistatin. Formalin-fixed lung sections were stained immunohistochemically with antibody to activin βA subunit. Representative micrographs, original magnification 400 x. (b) Airway epithelial activin A staining intensity in small-medium airways. Mean ± sem, n=3 mice/group.

Figure S6. Chronic allergen challenge causes loss of ActRIIA and ActRIIB immunoreactivity in airway epithelium. Mice were sensitised as described in Supplementary Figure 1b, and challenged with OVA, or OVA and follistatin; controls received saline instead of OVA or follistatin. Formalin-fixed lung sections were stained immunohistochemically with antibodies to ActRIIA and ActRIIB. Representative micrographs, original magnification 400 x. Airway epithelial ActRIIA (b) and ActRIIB (c) staining intensity in small-medium airways. Mean ± sem, n=3 mice/group.

References

1. Hardy CL, Kenins L, Drew AC, et al. Characterization of a mouse model of allergy to a major occupational latex glove allergen Hev b 5. *Am J Respir Crit Care Med.* 2003;**167**:1393-9.

2. Vremec D, Zorbas M, Scollay R, et al. The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. *J Exp Med.* 1992;**176**:47-58.

3. Hardy CL, O'Connor A E, Yao J, et al. Follistatin is a candidate endogenous negative regulator of activin A in experimental allergic asthma. *Clin Exp Allergy*. 2006;**36**:941-50.

4. O'Connor AE, McFarlane JR, Hayward S, et al. Serum activin A and follistatin concentrations during human pregnancy: a cross-sectional and longitudinal study. *Hum Reprod.* 1999;**14**:827-32.

Fig. S1

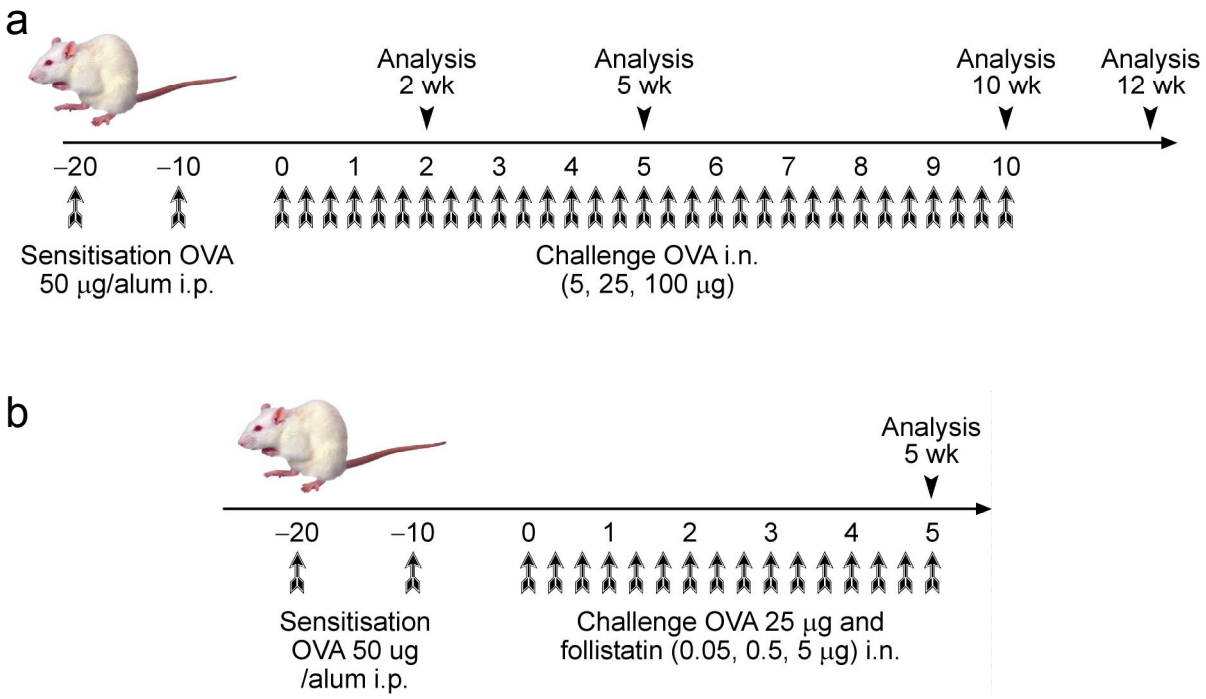

a
Analysis
2 wk
Analysis
5 wk
Analysis
10 wk
Analysis
12 wk
–20
–10
0
1
2
3
4
5
6
7
8
9
10
Sensitisation OVA
50 μg/alum i.p.
Challenge OVA i.n.
(5, 25, 100 μg)
b
Analysis
5 wk
–20
–10
0
1
2
3
4
5
Sensitisation
OVA 50 ug
/alum i.p.
Challenge OVA 25 μg and
follistatin (0.05, 0.5, 5 μg) i.n.

Fig. S2

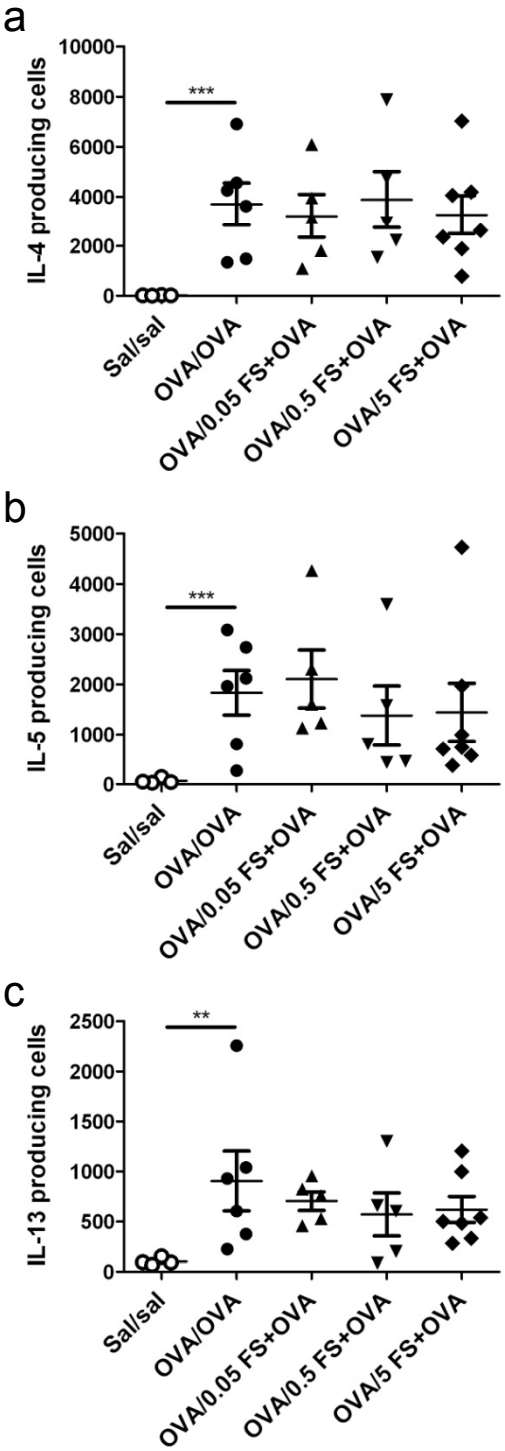

a
IL-4 producing cells
10000
8000
6000
4000
2000
0
***
Sal/sal
OVA/OVA
OVA/0.05 FS+OVA
OVA/0.5 FS+OVA
OVA/5 FS+OVA
b
IL-5 producing cells
5000
4000
3000
2000
1000
0
***
Sal/sal
OVA/OVA
OVA/0.05 FS+OVA
OVA/0.5 FS+OVA
OVA/5 FS+OVA
c
IL-13 producing cells
2500
2000
1500
1000
500
0
**
Sal/sal
OVA/OVA
OVA/0.05 FS+OVA
OVA/0.5 FS+OVA
OVA/5 FS+OVA

Fig. S3

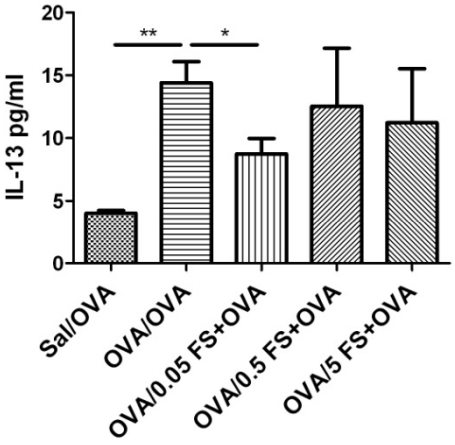

IL-13 pg/ml
20
15
10
5
0
**
*
Sal/OVA
OVA/OVA
OVA/0.05 FS+OVA
OVA/0.5 FS+OVA
OVA/5 FS+OVA

Fig. S4

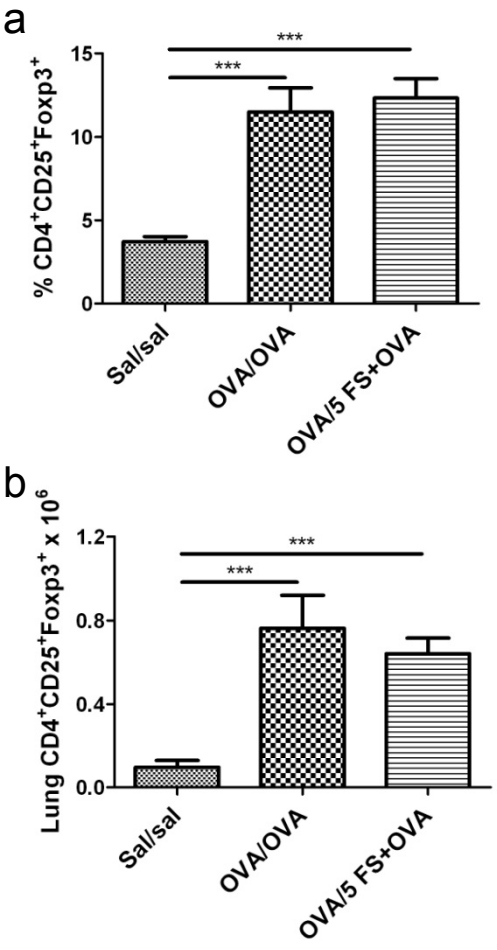

a
***
***
15
10
5
0
% CD4+CD25+Foxp3+
Sal/sal
OVA/OVA
OVA/5 FS+OVA
b
***
***
1.2
0.8
0.4
0.0
Lung CD4+CD25+Foxp3+ x 10^6
Sal/sal
OVA/OVA
OVA/5 FS+OVA

Fig. S5

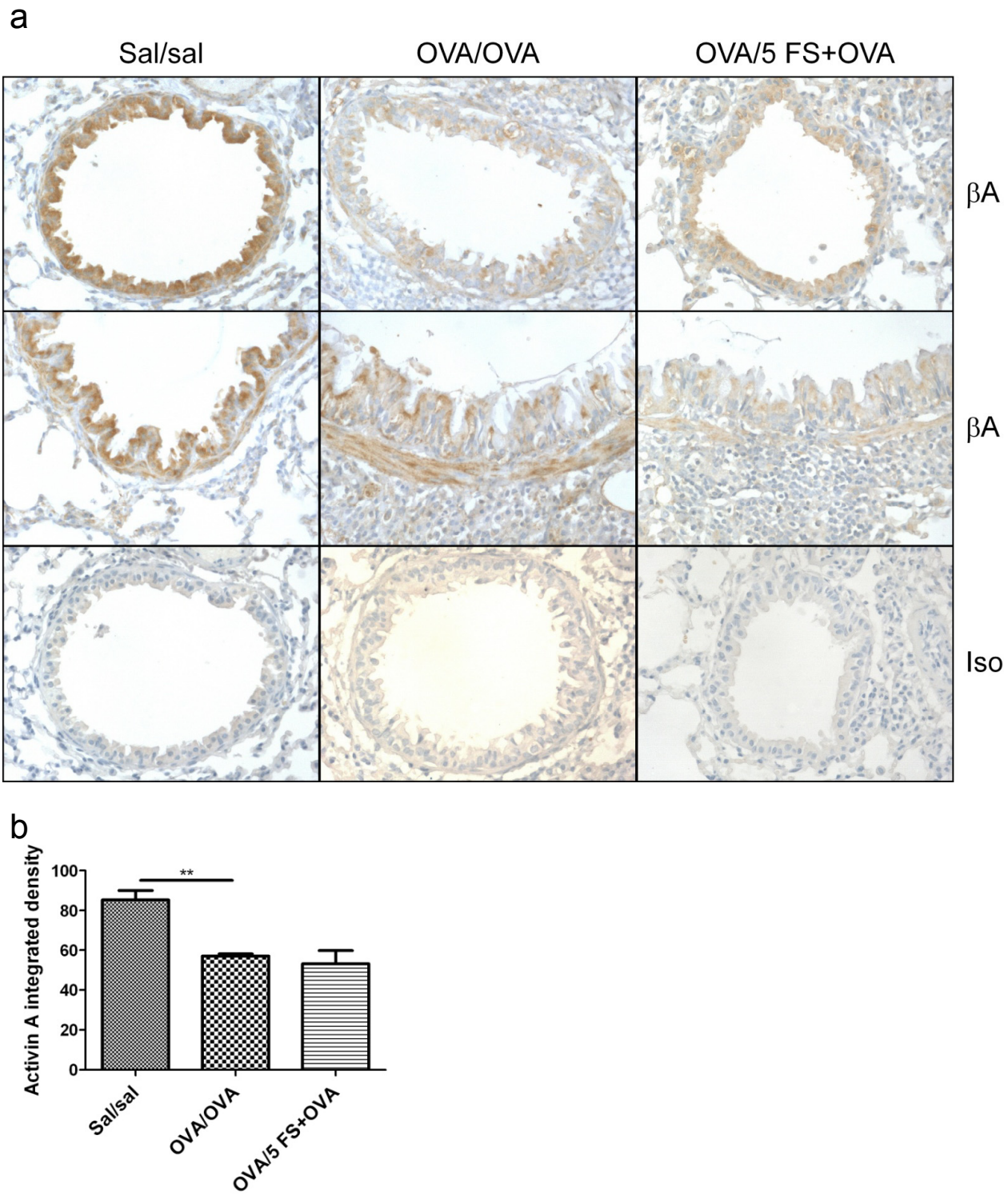

a
Sal/sal
OVA/OVA
OVA/5 FS+OVA
βA
βA
Iso
b
Activin A integrated density
100
80
60
40
20
0
**
Sal/sal
OVA/OVA
OVA/5 FS+OVA

Fig. S6

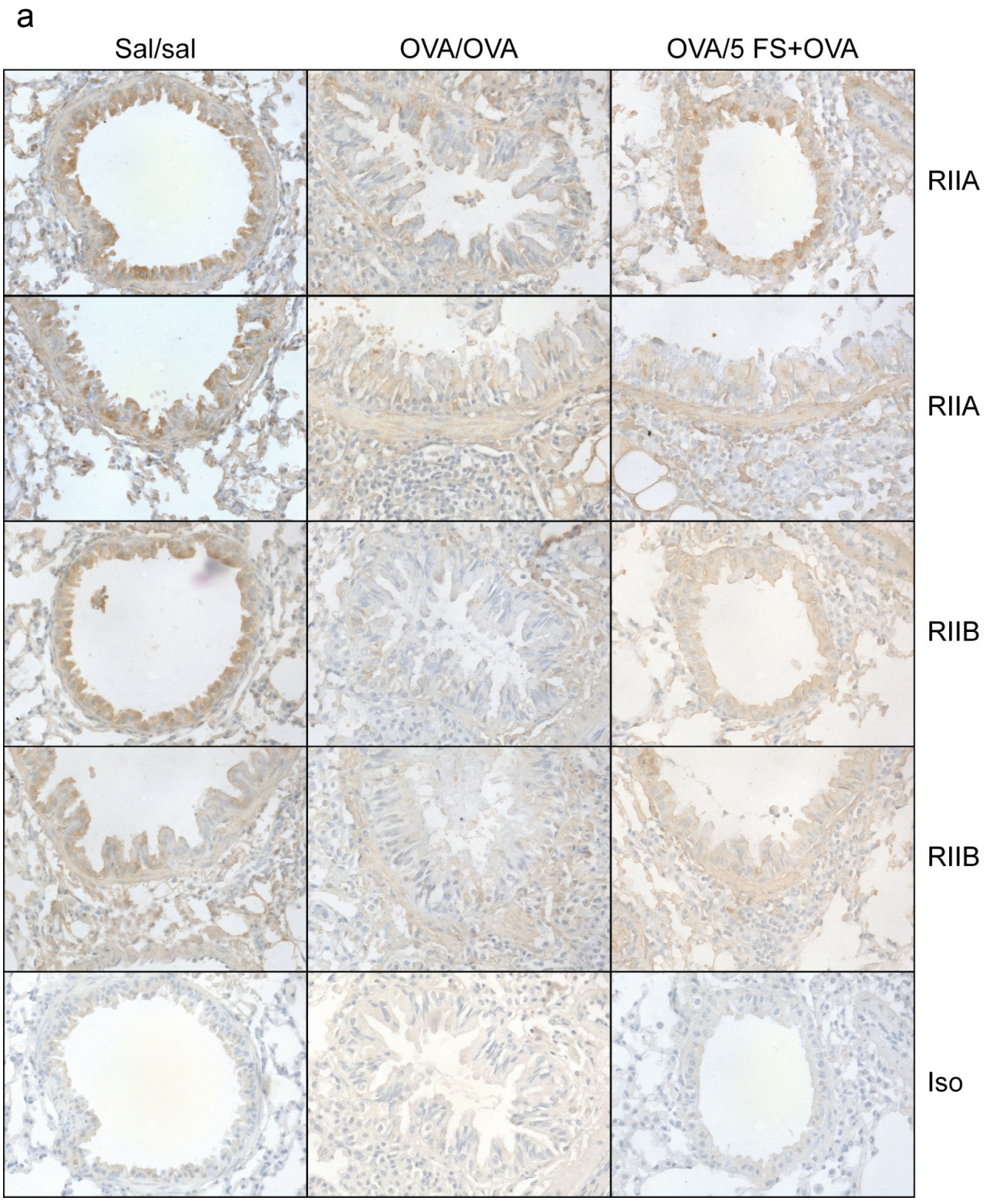
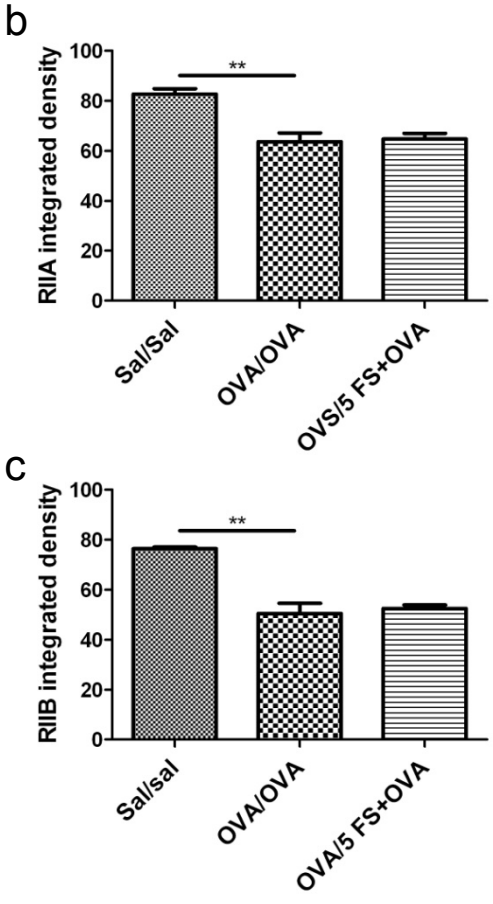


Fig. S6 cont.


b
RIIA integrated density
100
80
60
40
20
0
**
Sal/Sal
OVA/OVA
OVS/5 FS+OVA
c
RIIB integrated density
100
80
60
40
20
0
**
Sal/sal
OVA/OVA
OVA/5 FS+OVA