

ORIGINAL PAPER

Follistatin is a candidate endogenous negative regulator of activin A in experimental allergic asthma

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Clinical and Experimental Allergy

Summary

Background Activin A is a member of the transforming growth factor- β superfamily which is directly implicated in airway structural change and inflammation in asthma. *In vitro*, the biological effects of activin A are neutralized by the soluble binding protein follistatin.

Objective To determine the potential of endogenous follistatin to suppress activin A *in vivo* by analysing their relative tissue and kinetic compartmentalization during the effector phase of subchronic Th2-driven mucosal inflammation in a murine model of allergic asthma.

Methods Eosinophilic mucosal inflammation was elicited by triggering Th2 recall responses by antigen challenge in ovalbumin-sensitized BALB/c mice. The kinetics and distribution of activin A and follistatin protein were assessed in lung tissue and bronchoalveolar lavage fluid and measured in relation to airway eosinophilia, goblet cell metaplasia and Th2 cytokine production in mediastinal lymph nodes.

Results Follistatin was released concurrently with activin A suggesting it acts as an endogenous regulator: peak BAL concentrations coincided with maximal airway eosinophilia, and frequency of IL-4, IL-5 and IL-13 producing cells in mediastinal lymph nodes but induction lagged behind the onset of inflammation. Follistatin and activin A immunoreactivity were lost in airway epithelial cells in parallel with goblet cell metaplasia. Exogenous follistatin inhibited the allergen-specific Th2 immune response in mediastinal lymph nodes and mucus production in the lung.

Conclusion Follistatin is preformed in the normal lung and released in concert with activin A suggesting it serves as an endogenous regulator. Disturbance of the fine balance between activin A and its endogenous inhibitor follistatin may be a determinant of the severity of allergic inflammation or tissue phenotypic shift in asthma.

Keywords activin A, asthma, follistatin, inflammation, lung, Th2 cytokines

Submitted 23 September 2005; revised 4 April 2006; accepted 24 April 2006

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Introduction

Asthma is the most common chronic illness in children and adolescents, and is characterized by airway inflammation, airflow obstruction and bronchial hyper-reactivity [1]. Asthma may result from dysregulated immunity to common environmental aeroallergens, with Th2 type T cells and their secreted cytokines playing a central role in its pathogenesis [2–4]. The observation that transforming growth factor- β (TGF- β) plays important roles in immunomodulation and airway remodelling in asthma [5–7] has spurred interest in related TGF- β superfamily proteins.

Activins are members of the TGF- β family involved in a diverse array of cellular processes including growth/differentiation, matrix production and inflammation [8]. Activins A and B are homodimers of β_A and β_B subunits, respectively, while activin AB is a heterodimer of these subunits. Although there is some overlap in function of activin A and activin B, there is evidence supporting unique functions of these molecules.

Activin A is implicated directly in several inflammatory disorders including skin injury, inflammatory bowel disease, pulmonary and hepatic fibrosis and inflammatory

arthropathies [9, 10]. In the allergic asthmatic lung, mast cells are a major source of activin A [11], and activin A expression is increased in serum and CD4⁺ T cells from asthmatic patients [12]. Additionally, ligation of the high affinity IgE receptor on monocytes from atopic individuals causes activin A gene overexpression [13]. It is strongly suspected that activin A actively signals in murine asthma models where phosphorylated Smad 2, a TGF- β family signalling intermediate, and the activin receptor IB (ActRIB, also called ALK-4) are up-regulated in the lungs [14]. The emerging evidence therefore strongly supports a role for activin A in the pathogenesis of allergic airway inflammation.

Like TGF- β , activin A has both pro- and anti-inflammatory effects, with the net outcome possibly dependent on the local tissue concentration and modulatory effect of binding proteins [10]. Similar to TGF- β , activin signalling is also regulated at multiple levels including competition for receptor binding by structurally related molecules and inhibition by 'inhibitory' Smads. Distinct from TGF- β , activin A function is modulated by follistatin, which binds activin A with high affinity, blocking interaction with the activin A receptor complex and thereby neutralizing the biological actions of activin A [8]. Most recently, follistatin has been shown to inhibit neutrophilic and mononuclear cell inflammation and fibrosis in a bleomycin lung injury model [15], directly implicating follistatin as the dominant endogenous negative regulator of activin A.

To date, a detailed kinetic analysis of activin A and follistatin expression relative to markers of Th2 inflammation in asthma has not been performed. We investigated these relationships in a mouse model of ovalbumin (OVA)-induced acute allergic asthma. Our data predict that failure of follistatin to repress the activin axis *in vivo* would promote inflammation and airway remodelling in chronic asthma.

Materials and methods

Mice

Female BALB/c mice aged 7–8 weeks were obtained from Laboratory Animal Services (Adelaide, SA, Australia) and housed in the Alfred Medical Research and Education Precinct animal facility. All experimental protocols were approved by the precinct Animal Ethics Committee.

Immunization protocol

Mice were sensitized and challenged to OVA as described previously [16]; mice sensitized and challenged according to this protocol develop airways hyper-reactivity 24 h after the last challenge (data not shown). Mice were sensitized intraperitoneally with saline or OVA (50 μ g; Sigma-Aldrich, NSW, Australia) in alum (Reheis, NJ,

USA) on days –24 and –12, and challenged via intratracheal instillation with saline or OVA (25 μ g) on days 0, 2, 5 and 7. Mice were killed 24 h after each of the four challenges (days 1, 3, 6 and 8), and on days 9, 11, 14 and 17 (counted as days after the first intubation; Fig. 1a).

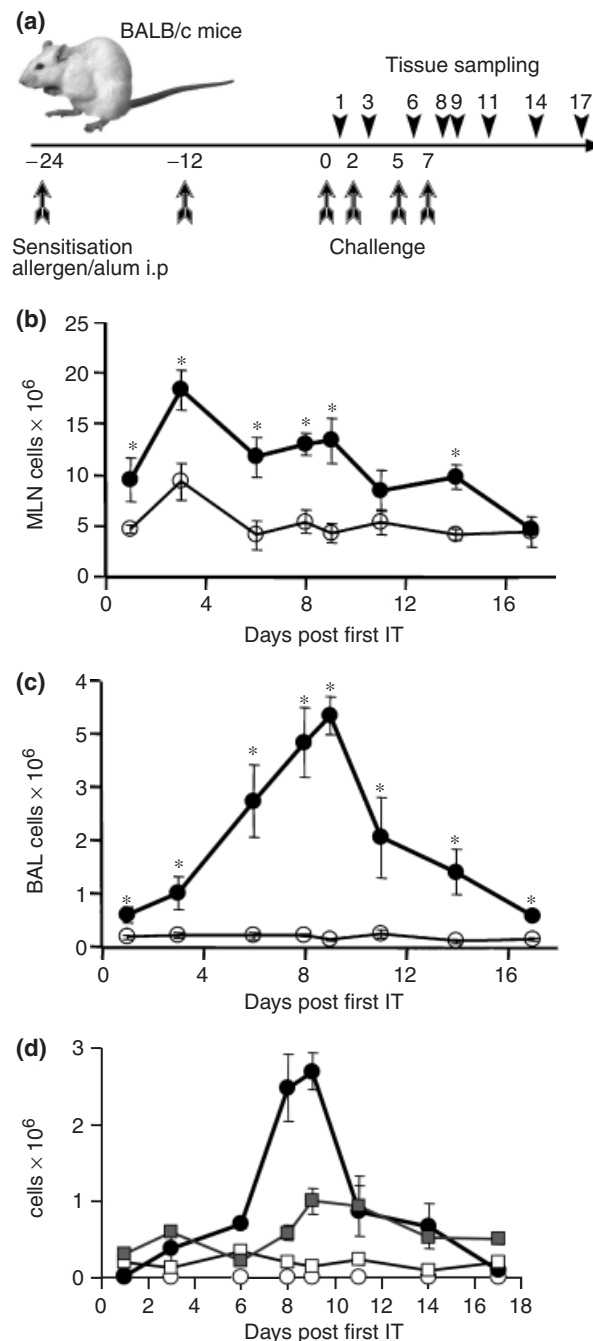


Fig. 1. Time course analysis of cell numbers and eosinophilia. Experimental protocol and sampling time-points (a), cell counts in mediastinal lymph node (MLN) (b) and bronchoalveolar lavage (BAL) (c). Total eosinophil (circles) and macrophage (squares) numbers in BAL (d). Open symbols, saline; closed symbols, OVA immunization. $n = 5-10$ mice per group per time-point, mean $\pm$ SEM. *Statistically significant difference between saline and OVA groups.

Follistatin administration

Mice were sensitized and challenged with saline or OVA (as above). Recombinant mouse follistatin (R&D Systems, Minneapolis, MN, USA; 769-FS, 0.05–1 µg in 0.1% BSA/saline) was administered intranasally (50 µL) 1 h before each challenge. Saline or OVA control groups did not receive follistatin. Mice were killed 24 h after the fourth challenge.

Bronchoalveolar lavage, differential counts and tissue sampling

Methods were as described previously [16]. Blood was collected from the inferior vena cava and serum collected by centrifugation. Bronchoalveolar lavage (BAL) was performed with 0.4 mL 1% fetal calf serum (FCS) in PBS followed by three further lavages of 0.3 mL. Viable mediastinal lymph node (MLN) lymphocytes and BAL leukocytes were counted in a hemocytometer. For differentials, BAL cytoposits were Giemsa-stained (Merck, Kilsyth, Victoria, Australia) and ≥ 200 cells identified by morphological criteria. For image analysis experiments mice were injected with heparin and killed via CO₂ asphyxiation before perfusion via the right ventricle with 10 mL freshly made 2% paraformaldehyde pH 7.3 in PBS. Lungs were paraffin-embedded and 3 µm sections stained with periodic acid-Schiff (PAS) for goblet cell quantitation.

Cytokine ELISPOT

ELISPOT plates (Millipore, North Ryde, NSW, Australia) were coated with IL-4 (BVD4-1D11), IL-5 (TRFK5; BD Pharmingen, San Jose, CA, USA) or IL-13 (MAB413; R&D Systems) antibody at 5 µg/mL in PBS overnight at 4 °C. Plates were blocked with 10% FCS in RPMI 1640 (Invitrogen, Mount Waverly, Victoria, Australia) for 1 h at 37 °C. Cells were resuspended in RPMI 1640 containing 2.5% FCS, 50 µM 2-mercaptoethanol (Sigma-Aldrich M-7522), penicillin-streptomycin-glutamine (Invitrogen 10378-016) and 10 mM HEPES. Blocking solution was discarded, and 0.5×10^6 cells in 100 µL added to the wells with 10 µL of appropriately diluted antigen. Following culture for 16 h at 37 °C, cells were discarded, plates soaked in distilled water for 10 min and washed. Biotinylated IL-4 (BVD6-24G2), IL-5 (TRFK4) (BD Pharmingen) or IL-13 (BAF413, R&D Systems) antibodies diluted in PBS were added for 1.5 h. Plates were washed and ExtrAvidin® – alkaline phosphatase (Sigma-Aldrich E-2636) added for 1.5 h. Washes were in 0.05% Tween 20/PBS followed by PBS. Reaction product was developed (Bio-Rad, Regents Park, NSW, Australia 170-6432), plates washed in water and air dried. Plates were read on an ELISPOT reader (Autoimmun Diagnostika, Strassberg, Germany).

Activin ELISA and follistatin radioimmunoassay

Activin A was measured using a specific ELISA [17] according to the manufacturer's instructions (Oxford Bio-Innovations, Upper Heyford, Oxfordshire, UK) with some modifications as described previously [18]. Follistatin concentrations were measured in serum and BAL fluid (BALF) using a discontinuous radioimmunoassay as described previously [18]. Note that 1 nM activin A = 25 ng/mL, 1 nM follistatin = 35–45 ng/mL (variably glycosylated and different isoforms). The activin A antibody (E4) used for both the ELISA and immunohistochemistry was obtained from Professor Nigel Groome, Oxford Brookes University, UK. The follistatin antibodies (#204 and 2E6) used for the immunoassay and immunohistochemistry were developed 'in house' and have been raised against purified native bovine follistatin or human recombinant follistatin. The assay buffer for the BALF samples was 0.05% BSA/PBS. Mouse serum and BALF sample dilutions were parallel to a standard curve (data not shown). The limit of activin A detection for both serum and BALF samples was 0.01 ng/mL. The limit of follistatin detection was < 0.5 and < 1.04 ng/mL for serum and BALF samples, respectively.

Activin and follistatin 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 1000 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% H₂O₂ for 10 min, and non-specific binding blocked for 1 h (CAS block with 10% normal rabbit serum, Zymed Laboratories, South San Francisco, CA, USA; 00-8120). Sections were incubated with antibodies specific for the activin β_A subunit (E4) or follistatin (2E6) at 10 µg/mL overnight at 4 °C. After washing, slides were incubated in rabbit anti-mouse-IgG_{2b}-peroxidase (Zymed 61-0320) or IgM-peroxidase (Zymed 61-6820) diluted 1 : 500 for 2 h, for activin A and follistatin primary antibodies, respectively. Slides were washed in Tris-buffered NaCl (TBS) 0.05% Tween-20 pH 7.5, then MilliQ H₂O. Reaction product was developed with 3,3'-diaminobenzidine tetrahydrochloride substrate kit (Zymed 00-2014), and sections counterstained in Harris's hematoxylin. All wash steps were in TBS/0.05% Tween-20. Antibodies were diluted in 1% BSA/TBS. Non-immune mouse antibody of the appropriate immunoglobulin isotype was used for negative controls (Zymed 02-6800 and 02-6300 for IgM and IgG_{2b}, respectively).

Image analysis

Sections were analysed at $\times 200$ or $\times 400$ magnification using appropriately calibrated Image-Pro Plus 5.0 software (Media Cybernetics, Silver Spring, MO, USA). The

linear basement membrane length was measured (mm), and the number of activin A-, follistatin- or PAS-positive cells in the airway counted, yielding number of positive cells/mm basement membrane. Ten airways per mouse were assessed, except for the follistatin inhibition experiments where all airways in the lung sections from each mouse were assessed (50–100 airways per mouse). Semi-quantitative grading of mucus production by airways was performed blinded using a scale of 1–5, where 1 = negligible staining, and 5 is > 90% of airways showing high frequency and/or volume (cell size) of PAS-positive epithelial cells.

Statistical analysis

Statistics were determined using SPSS 12.0.1 software. The Mann–Whitney U test was used, with a *P* value ≤ 0.05 considered statistically significant. For analysis of IL-4, IL-5 and mucus cell frequency in the follistatin inhibition experiments, data were assessed for normality and log-transformed before analysis by ANOVA.

Results

Kinetics of the pulmonary allergic inflammatory response

Groups of mice were analysed after each challenge, and at various times after the final (fourth) challenge (Fig. 1a). MLN cell counts peaked after the second challenge in immunized mice, and gradually returned to pre-challenge levels by day 17 (Fig. 1b). In contrast, BAL cell numbers peaked at day 9, with numbers returning to pre-challenge levels by day 17 (Fig. 1c). Differential analysis of BAL cells showed that eosinophil frequency and total numbers peaked at days 8 and 9, declining rapidly thereafter (Fig. 1d). Macrophages numbers in BAL showed an approximate twofold increase at days 9–11 (Fig. 1d). There was an approximate twofold increase in BALF IL-5 concentration in OVA-immunized mice at day 8, although no difference was seen at other times; no significant differences in BALF IL-4 concentrations were seen at any time (data not shown). In contrast, ELISPOT analysis of IL-4 and IL-5 production by MLN showed an increased frequency of IL-4- and IL-5-producing cells immediately after the first challenge until day 14, 7 days after the final challenge. The kinetics of the IL-4 and IL-5 response were very similar, with peak frequency seen at day 6, and a broad shoulder of moderate frequency persisting until day 14 (Figs 2a and b). The frequency of IL-4 secreting cells was always greater than that for IL-5 at the corresponding time-point. IL-13 production peaked at day 8, but in contrast to the results for IL-4 and IL-5, the frequency of IL-13 producing cells was still significantly elevated at day 17 (Fig. 2c). Collectively these data indicate that the

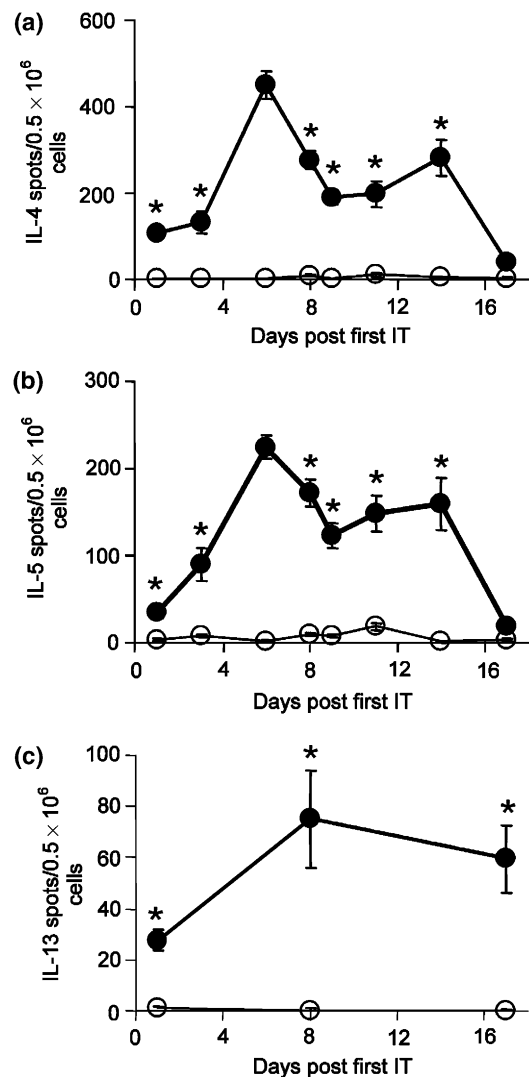


Fig. 2. Time-course analysis of IL-4, IL-5 and IL-13 production by draining lymph node (LN) cells. Frequency of IL-4- (a), IL-5- (b) and IL-13- producing (c) mediastinal LN (MLN) cells as measured by ELISPOT. Open circles, saline; closed circles, OVA immunization. *n* = 5–10 mice per group per time-point, mean $\pm$ SEM. *Statistically significant difference between saline and OVA groups.

pulmonary immune response peaked after three to four challenges (days 6–8) and gradually subsided thereafter.

Activin and follistatin concentrations in bronchoalveolar lavage fluid

BALF was collected at each time-point and activin A and follistatin concentrations determined. Activin A was barely detectable in BALF from control mice at all times. In contrast, in OVA-sensitized mice activin A concentrations were elevated after one OVA challenge, and peaked on days 6 and 8 (after three to four challenges). The activin A concentration dropped rapidly between days 9 and 11, and had returned to control levels by day 17

(10 days after final challenge) (Fig. 3a). There was also a clear increase in follistatin concentrations in BALF at days 6 and 8 (Fig. 3b), thus paralleling the activin A kinetics. Activin A and follistatin levels lagged behind the initial infiltration of inflammatory cells but coincided with peak inflammation. No obvious fluctuations in activin A or follistatin concentrations in serum were seen at any time-point (activin A, < 0.2 ng/mL for both saline and OVA groups; follistatin, 3–5 ng/mL for both saline and OVA groups).

Activin, follistatin and mucus localization/production in the inflamed lung

Bronchial epithelium from control mice showed strong and uniform activin A staining at all times. Pneumocytes expressed activin A at low density, and occasionally strong localization by macrophages was also seen (Fig. 4a). Mice which had received one OVA challenge (day 1) had airway morphology and localization of activin A essentially indistinguishable from controls, although mild inflammation was occasionally observed (Fig. 4c). In contrast, after three to four OVA challenges there was a dramatic loss of activin A staining in airway epithelium, accompanied by extensive peribronchial and perivascular inflammation and epithelial hypertrophy. Discrete macrophages in the inflammatory infiltrate strongly expressed

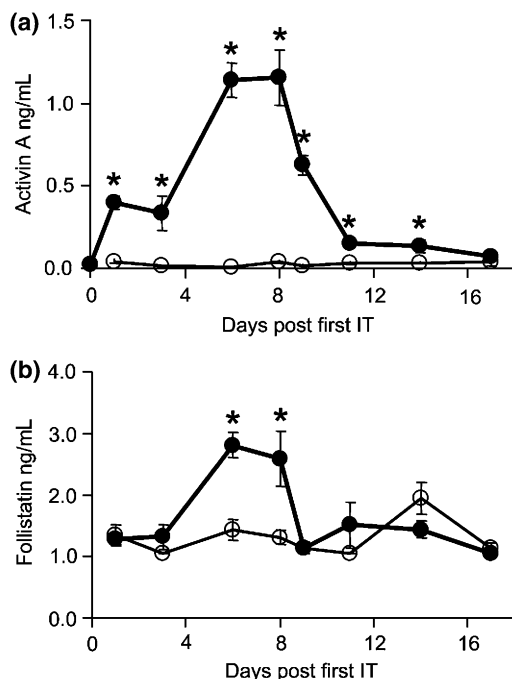


Fig. 3. Time-course analysis of bronchoalveolar lavage fluid (BALF) activin A (a) and follistatin (b) concentrations. Open circles, saline; closed circles, OVA immunization. $n = 5$ –10 mice per group per time-point, mean $\pm$ SEM. *Statistically significant difference between saline and OVA groups.

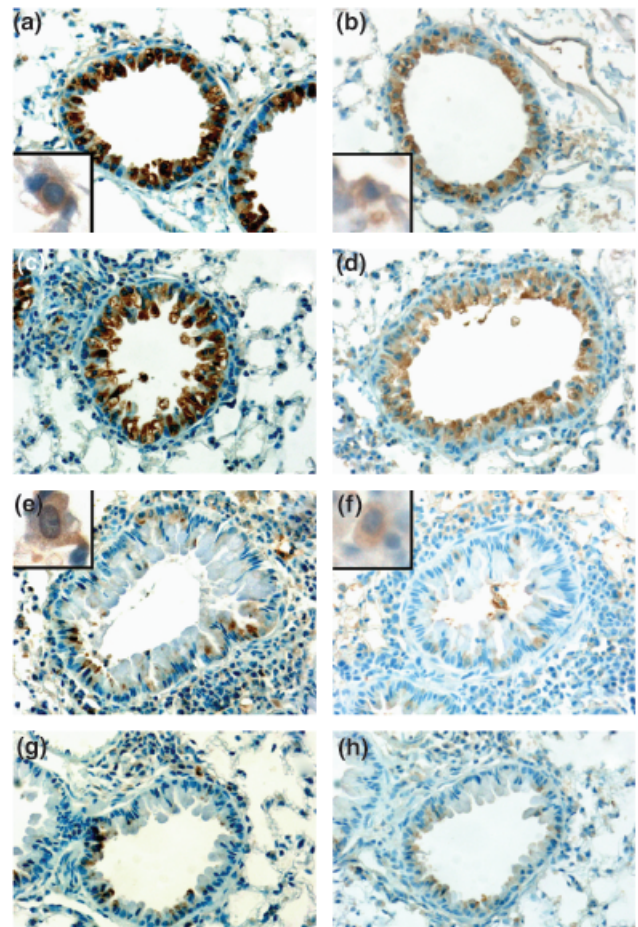


Fig. 4. Activin A and follistatin localisation in airways. Activin A localisation in saline-treated control mice remained stable at all time-points (a). Activin A localisation at days 1 and 8 (c and e; after one and four OVA challenges, respectively), and day 17 (g; 10 days after the final OVA challenge). Follistatin localisation in control lung (b) and at days 1, 8 and 17 (d, f and h, respectively). Inset shows a positive macrophage, the main non-epithelial source found in the study. Immunoperoxidase, hematoxylin counterstain, original magnification $\times 400$. Representative micrographs from 11 to 16 mice per time-point.

activin A (Fig. 4e). There were no differences in the frequency of activin A-expressing macrophages between control and inflamed lung (data not shown). The structural changes and associated loss of activin A persisted through days 9–11 (data not shown). By day 17 inflammation, airway thickening and epithelial hypertrophy had subsided although most airways continued to show a marked loss of activin A staining (Fig. 4g).

The localization pattern for follistatin was also examined. Control mice had strong and uniform follistatin localization in bronchial epithelium at all time-points. Follistatin was expressed weakly by pneumocytes, although strong staining by macrophages was occasionally noted (Fig. 4b). After one OVA challenge the pattern resembled that of control mice (Fig. 4d). However, after three to four challenges the majority of airways showed a

near complete loss of follistatin staining. Pneumocytes expressed follistatin at increased density and discrete lymphoblasts/macrophages were positive (Fig. 4f). As with activin A, there were no differences in the frequency of follistatin-expressing macrophages between control and inflamed lung (data not shown). These changes persisted through days 9–11 (data not shown). Follistatin localisation remained markedly reduced in the majority of airways at day 17 (Fig. 4h).

Metaplasia of airway epithelium to goblet cells is a common feature of asthma in mouse models and the human. We investigated the possible relationship between loss of activin A/follistatin localization by airway epithelium and acquisition of a mucus-secreting phenotype. Control mice had a complete absence of mucus-producing cells in the airways at all time-points (Fig. 5a). After one OVA challenge mucus-producing goblet cells were very infrequently seen (Fig. 5b). However, after four challenges the number of airways containing goblet cells was dramatically increased, although the frequency of positive cells within individual airways was variable (Fig. 5c). The number of airways containing goblet cells had decreased by day 17 (10 days after the final challenge), although it was still clearly elevated compared to controls, and variation between individual airways was again noted (Fig. 5d).

Quantitative analysis of activin, follistatin and mucus localization/production by airway epithelium

Image analysis was performed at three key time-points to more precisely investigate the dynamics of activin A, follistatin and mucus localization in the airways. After one OVA challenge the frequency of activin A-positive

epithelial cells was nearly identical to controls (130/mm basement membrane; Fig. 6a). However, after four challenges (day 8) the frequency of activin A-positive airway epithelial cells was significantly decreased (51/mm basement membrane). This change was still observed 10 days after the final challenge, although the frequency of activin A-positive cells had increased slightly (70/mm basement membrane). Similar changes were observed when follistatin-expressing airway epithelial cells were analysed. The frequency of follistatin-positive cells was approximately 100/mm basement membrane in both control and OVA-challenged mice at day 1. However, after four OVA challenges the frequency of follistatin-positive cells was significantly decreased (43/mm basement membrane),

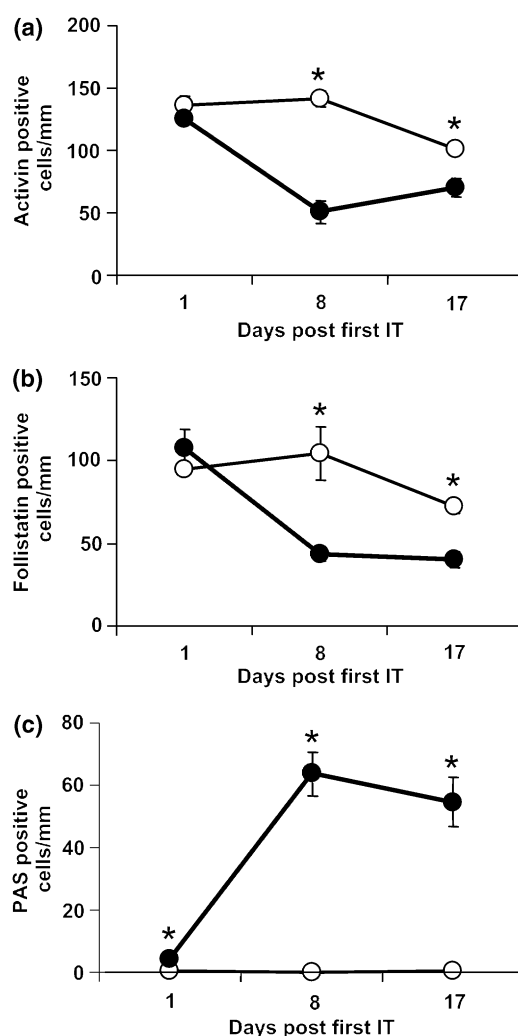


Fig. 6. Frequency of activin A-, follistatin- and mucus-expressing airway epithelial cells. Quantitative image analysis of activin A-, follistatin- and periodic acid-Schiff (PAS)-positive cells per mm basement membrane (a–c, respectively). Activin A- and follistatin-specific immunoperoxidase (a and b) and PAS (c). Open circles, saline; closed circles, OVA immunization. $n = 6$ mice per group per time-point, mean $\pm$ SEM. *Statistically significant difference between saline and OVA groups.

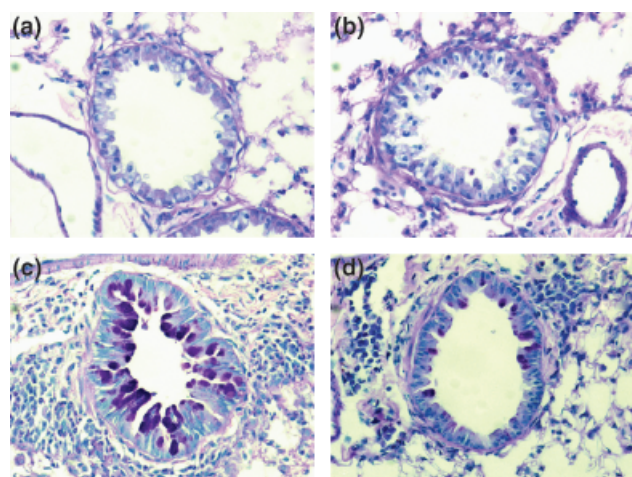


Fig. 5. Mucus production by bronchial epithelium. Airway epithelium from control mice did not contain mucus-producing goblet cells (a). Goblet cells in airways from OVA mice at days 1, 8 and 17 (b–d, respectively). Periodic acid-Schiff (PAS), original magnification $\times 400$. Representative micrographs from 11 to 16 mice per time-point.

and an almost identical frequency was observed 10 days after the final challenge (Fig. 6b). There was a slight drop in the frequency of activin A- and follistatin-positive cells in control mice between days 8 and 17; this change was statistically significant for activin A only.

We performed a parallel quantitative analysis of mucus-secreting cell frequency. Goblet cells were almost undetectable in control mice at all time-points, while their frequency was very slightly elevated in OVA-challenged mice at day 1 (4/mm basement membrane) (Fig. 6c). However, by day 8 goblet cell frequency in OVA-challenged mice was dramatically elevated (64/mm basement membrane). This frequency had dropped marginally by day 17 but was still well above control values (Fig. 6c). The mucus-producing response was temporally linked to increased IL-13 production in draining LN (Fig. 2c), consistent with the key role for this cytokine in mucus production in allergic asthma [19, 20]. Collectively these findings reveal an inverse relationship between airway epithelial activin A/follistatin localization and metaplasia to goblet cells.

Effect of exogenous follistatin

To directly test whether follistatin can inhibit an allergen-specific Th2 response saline (control) or OVA sensitized mice received recombinant mouse follistatin before each of four saline or OVA challenges, respectively. Exogenous follistatin resulted in approximately 20% lower activin A concentrations in BALF (1.08 ± 0.08 vs. 0.88 ± 0.09 ng/mL for OVA vs. OVA+follistatin groups, respectively, $P=0.08$). Furthermore, exogenous follistatin caused decreased MLN cell numbers (Fig. 7a), and decreased frequency of cells producing IL-4 and IL-5 (Figs 7b and c) 24 h after the fourth challenge. Semi-quantitative blinded grading of the frequency and volume (cell size) of mucus-producing airway cells yielded mean scores of 3.36 ± 0.28 vs. 2.0 ± 0.16 ($P=0.01$), and 3.0 ± 0.26 vs. 1.4 ± 0.29 ($P < 0.01$), for OVA vs. OVA+follistatin mice, respectively. Additionally, quantitative image analysis of all airways in the lung sections showed that follistatin decreased the frequency of mucus-producing airway epithelial cells (Fig. 7d). However, total BAL eosinophils were only slightly decreased in the OVA+follistatin groups (data not shown). Saline immunized groups had negligible IL-4, IL-5 or mucus production, and this was unaffected by exogenous follistatin. Overall these findings suggest that follistatin can inhibit the establishment of an allergen-specific Th2 recall response in mice.

Discussion

This study demonstrates that production of activin A and follistatin is dynamically regulated during the development of experimental allergic asthma in the mouse. There

was a dramatic loss of activin A and follistatin localization in the airway epithelium coinciding with increased activin A and follistatin concentrations in the BALF. Further, activin A and follistatin concentrations in BALF were closely related to the magnitude of the Th2 inflammatory response (airway eosinophils, goblet cell metaplasia and Th2 cytokine production). Histological assessment and quantitative image analysis demonstrated that the loss of activin A and follistatin from airway epithelial cells coincided with their metaplasia to a mucus-secreting phenotype. Normal airway epithelium is known to express activin A [21], and our findings now show that this is also true for follistatin. We report for the first time altered compartmentalization of activin A and follistatin in the lung during the evolution of a Th2-type pulmonary immune response. We also show that exogenous follistatin attenuates the development of allergic pulmonary inflammation.

Activin A and follistatin are thought to play key early roles in inflammatory responses. Studies in sheep and mice injected with lipopolysaccharide (LPS) demonstrate very rapid increases in serum activin A concentrations (45–60 min), before the rise in pro-inflammatory cytokines TNF- α , IL-6 and IL-1 β , while follistatin concentrations peak later at 5–12 h ([22, 23] and D. J. Phillips, unpublished). These findings are important because they indicate that activin A and follistatin are regulated consistently across different species. However, follistatin injection 0.5 h before LPS injection inhibits the activin A-induced rise in TNF- α and IL-1 in the mouse (D. J. Phillips, unpublished), suggesting that excess follistatin can attenuate a subsequent inflammatory response. This relationship is supported by the recent findings that follistatin inhibits bleomycin-induced fibrosis in rats [15], and experimental colitis in mice [24]. We observed that maximal BALF follistatin concentrations were coincident with maximal activin A concentrations. This finding is consistent with the role of follistatin as a protein which neutralizes activin A, possibly as a homeostatic mechanism to dampen activin A-mediated signalling [8]. The activin A and follistatin assays used by us detect total (i.e. free and bound) protein. This, together with the fact that follistatin can bind to heparin sulphate proteoglycans in the extracellular matrix means that it is unclear what proportion is free to interact with activin A. Nevertheless, activin A concentrations increased approximately 60-fold at the peak of the response whereas follistatin concentrations increased two to threefold, suggesting that there is a relative excess of activin A in the BALF. It should be noted, however, that in the absence of an activin A bioassay suitable for use with serum or BAL we cannot categorically state that we had increased activin A bioactivity. We also demonstrated that intranasally instilled follistatin inhibits the generation of an allergen-specific Th2 response in the lung-draining LN and mucus

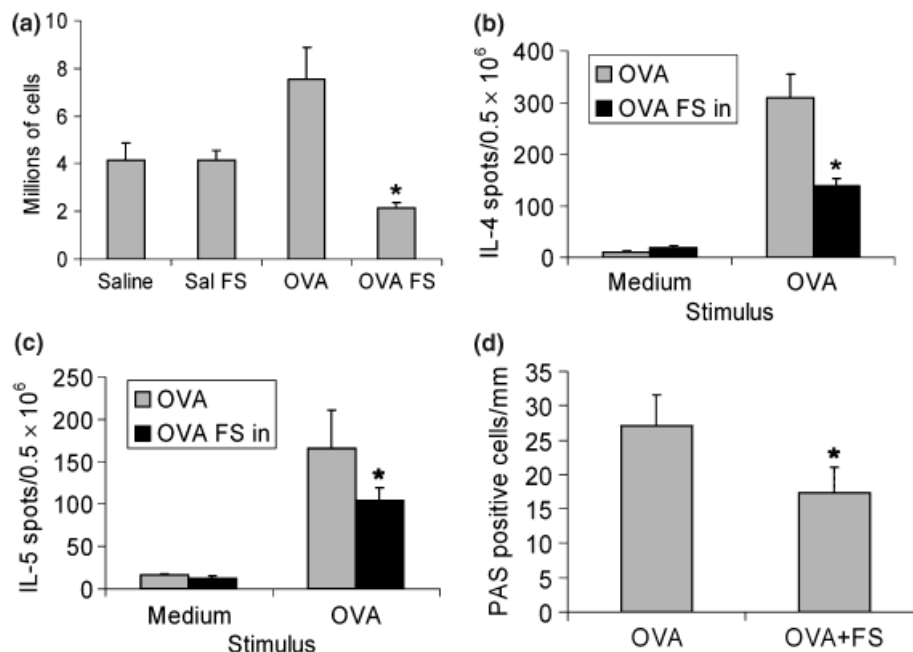


Fig. 7 Effect of exogenous follistatin on cell counts, IL-4 and IL-5 production in draining lymph node (LN), and frequency of mucus-producing airway epithelial cells 24 h after the fourth challenge. Total LN cell counts (a), frequency of IL-4- (b) and IL-5 (c) -producing cells in the LN, and frequency of periodic acid-Schiff (PAS)-positive cells/mm epithelial basement membrane (d). $n = 5-8$ mice per group, mean $\pm$ SEM, representative of three independent experiments. *Statistically significant difference between ovalbumin (OVA) and OVA + follistatin groups.

production in the lung. Collectively these findings suggest that exogenous follistatin may be able to ameliorate or prevent asthma pathology. We are currently investigating whether follistatin can inhibit the full spectrum of asthma symptoms in our mouse model.

The parallel decreased activin A and follistatin localization by bronchial epithelium and increased concentrations in BALF suggest that activin A and follistatin (possibly pre-stored) is released into the surrounding tissue during the inflammatory response. The effects of these cytokines are presumably polarized towards cells in the submucosa/parenchyma, and their presence in BALF may represent 'spillover' into the airway lumen. These changes noted for activin A mirror those seen for TGF- β , which is down-regulated in asthmatic bronchial epithelial cells [25], yet elevated in BALF from atopic asthmatics [26]. Others have similarly observed increased BALF activin A concentrations following OVA challenge at a single time-point, with mast cells proposed as the major source [11]. Macrophages can produce activin A [21], and we observed occasional activin A- and follistatin-expressing macrophages in the lung infiltrates (Fig. 4). BALF activin A concentrations increased 60-fold at days 6-8, while BAL macrophage numbers were low and increased twofold at days 9-11. These differences in timing and the relative fold-increases are not consistent with macrophages being a major source of BALF activin A. Our experiments show that activin A (and follistatin) expression by airway epithelium is progressively down-regu-

lated between days 6 and 8, concurrent with the appearance of activin A and follistatin in BALF. Additionally, in the normal lung 95-100% of epithelial cells express activin A (and follistatin), whereas positive macrophages are relatively infrequent (Figs 4a and b). Collectively our data are consistent with airway epithelial cells being the dominant source of activin A and follistatin in the BALF, with a minor contribution from macrophages. As indicated above, activin A is released in the early phases of LPS-induced inflammation. A similar early-phase release of activin A in asthma suggests that it may play a key role in driving and/or directing the severity of the ensuing pathology.

IL-4 and IL-13 trigger TGF- β release from asthmatic bronchial epithelium [27]. Additionally, IL-5 plays a key role in modulating eosinophil-derived TGF- β production in the airways via regulation of eosinophil numbers [28, 29]. Given the numerous overlaps in the biology of activin A and TGF- β function and signalling pathways [7, 8, 30], it is possible that activin A production is regulated by cytokines such as IL-4, IL-5 and IL-13; indeed it was recently shown that IL-13 induces activin A secretion from human lung fibroblasts [12]. This idea is consistent with the finding that maximal activin A concentration in BALF coincided with maximal eosinophil numbers, goblet cell metaplasia and frequency of draining LN cells producing IL-4, IL-5 and IL-13. However, we did not see activin A or follistatin immunoreactivity in eosinophils, arguing that eosinophils are not a major

source of activin A or follistatin. Conversely, activin A may regulate Th2-mediated events, as supported by the observation that activin A enhances IgE production from IL-4-stimulated human peripheral blood mononuclear cells [31].

Our observations of decreased activin A and follistatin localization by airway epithelium at the peak of the inflammatory response are superficially inconsistent with the increased expression of phosphorylated Smad 2 and the type I receptor ALK-4 in bronchial epithelium from OVA-challenged mice [14]. However, activin A/TGF- β signalling is regulated by diverse mechanisms including level of receptor expression, competition for receptor binding via ligand traps such as follistatin, and inhibitory Smads [8, 30]. Owing to these complex regulatory mechanisms, attempting to draw a direct connection between expression of TGF- β family type I and II receptors or signalling pathways and their ligand is inappropriate.

In conclusion, our findings demonstrate that dramatic changes in activin A and follistatin production occur in experimental allergic asthma. A pronounced decrease in activin A and follistatin localization in bronchial epithelium was kinetically related to increased activin A and follistatin concentrations in BALF. These alterations were closely related to the magnitude of the Th2 inflammatory response, as assessed by BAL eosinophil numbers and Th2 cytokine production in lung-draining lymph nodes. The changes in activin A and follistatin expression in bronchial epithelium were temporally associated with metaplasia to goblet cells, and it is tempting to speculate that a mechanistic relationship between activin expression and hypersecretion may exist. Our findings suggest that bronchial epithelium stores activin A which can be liberated into the submucosa upon initiation of allergic airway inflammation (possibly due to IL-4, IL-5 and IL-13 production), and that this may contribute to airway inflammation and remodelling. Inflammatory cells such as macrophages and mast cells may also release activin A thereby amplifying this response [11, 21, 32]. Our data suggest that endogenous follistatin is a negative regulator which dampens allergic airway inflammation, but does not prevent its induction. However, exogenous follistatin inhibits the generation of an allergen-specific immune response in the lung-draining LN and mucus production in the lung. Collectively, our findings underscore the notion that the activin–follistatin axis plays an important role in the pathogenesis of asthma and other chronic inflammatory lung diseases, and raise the possibility for therapeutic intervention with follistatin.

Acknowledgements

We thank Prof John Wilson and Dr Tiffany Bamford (Monash University) for helpful discussion. We thank Dr Michael Bailey for statistical advice (Department of

Epidemiology & Preventive Medicine, Monash University). This research was funded by Monash University Small Grants, the Cooperative Research Centre for Asthma and the National Health & Medical Research Council of Australia.

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