

Interleukin-13 Regulates Secretion of the Tumor Growth Factor- β Superfamily Cytokine Activin A in Allergic Airway Inflammation

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Activin A is a member of the TGF- β superfamily and plays a role in allergic inflammation and asthma pathogenesis. Recent evidence suggests that activin A regulates proinflammatory cytokine production and is regulated by inflammatory mediators. In a murine model of acute allergic airway inflammation, we observed previously that increased activin A concentrations in bronchoalveolar lavage (BAL) fluid coincide with Th2 cytokine production in lung-draining lymph nodes and pronounced mucus metaplasia in bronchial epithelium. We therefore hypothesized that IL-13, the key cytokine for mucus production, regulates activin A secretion into BAL fluid in experimental asthma. IL-13 increased BAL fluid activin A concentrations in naive mice and dose dependently induced activin A secretion from cultured human airway epithelium. A key role for IL-13 in the secretion of activin A into the BAL fluid during allergic airway inflammation was confirmed in IL-13-deficient mice. Eosinophils were not involved in this response because there was no difference in BAL fluid activin A concentrations between wild-type and eosinophil-deficient mice. Our data highlight an important role for IL-13 in the regulation of activin A intraepithelially and in BAL fluid in naive mice and during allergic airway inflammation. Given the immunomodulatory and fibrogenic effects of activin A, our findings suggest an important role for IL-13 regulation of activin A in asthma pathogenesis.

Keywords: activin A; IL-13; inflammation; lung; knockout mice

Activin A, a member of the TGF- β cytokine superfamily, is implicated in inflammation and tissue repair processes (1, 2). In particular, expression of activin A and its type I and type II receptors is differentially regulated in human asthma and experimental asthma models (3–5). Activin A promotes inflammation and tissue fibrosis in the lung and proliferation of human smooth muscle cells *in vitro* (6, 7). In a chronic allergen challenge model, mice lacking Smad 3, a signaling intermediate common to the TGF- β and activin A signaling pathways, have decreased peribronchial fibrosis and smooth muscle deposition, key characteristics of airway remodeling (8). Together, these findings are consistent with an important role for activin A in the pathogenesis of asthmatic airway inflammation and remodeling.

CLINICAL RELEVANCE

Our results highlight an important role for IL-13 in the regulation of the TGF- β superfamily cytokine activin A in allergic airway inflammation. Our findings reinforce the idea that activin A plays an important role in asthma pathogenesis and suggest that targeting this pathway may hold therapeutic potential.

The biological effects of activin A are tightly regulated at the level of the receptor complex and by a variety of activin-binding proteins (9). Activin A is produced very early during LPS-induced systemic inflammation and regulates expression of proinflammatory cytokines (10, 11). In turn, the modulatory effects of activin A are regulated by its high-affinity binding protein, follistatin, in LPS-induced endotoxemia, colonic inflammation, and acute allergic airway inflammation (3, 11, 12). Furthermore, activin A secretion from bone marrow-derived stromal fibroblasts is enhanced by IL-1 β and LPS and inhibited by IFN- γ (13). These findings indicate that activin A regulates, and is regulated by, inflammatory mediators.

IL-13 plays a major role in the pathogenesis of asthma by inducing mucus hypersecretion, airway hyperreactivity, eosinophil recruitment, and IgE class switching (14, 15). The direct action of IL-13 on airway epithelial cells induces airway hyperreactivity and mucus production (16). IL-13 induces TGF- β secretion from human bronchial epithelial cells (17, 18), and IL-13 overexpression in the lung induces airway remodeling largely mediated by the induction of TGF- β (19). We previously demonstrated strong activin A immunoreactivity in airway epithelial cells in naive mice, which decreased in parallel with increasing bronchoalveolar lavage (BAL) fluid activin A concentrations in mice with allergic airway inflammation, suggesting that airway epithelial cells are a major source of activin A in allergic airway inflammation (3). In this model, we also showed that peak BAL fluid activin A concentrations coincide with maximal airway epithelial mucus metaplasia and Th2 cytokine production in lung-draining lymph nodes (LNs) (3). These findings, together with the parallels between TGF- β and activin A signaling pathways, suggest a pivotal role for IL-13 in regulation of activin A expression in the lung. In the current study, we investigated the effect of IL-13 on activin A expression in naive mice and on cultured human bronchial epithelium. The effect of IL-13 on activin A during acute allergic airway inflammation was further examined in IL-13-deficient mice. Our findings indicate that the Th2 cytokine IL-13 plays an important role in the regulation of activin A in allergic asthma. Given roles for activin A in immunomodulation and tissue remodeling, our findings implicate the IL-13-activin A axis in asthma pathogenesis.

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MATERIALS AND METHODS

Animals

Female BALB/c mice, 6 to 7 weeks age, were obtained from Laboratory Animal Services (The University of Adelaide, South Australia). BALB/c.B6129.IL13^{tm1} knockout (KO) and BALB/c.IL-5^{tm1}Eotaxin^{tm1} double knockout (DKO) mice were obtained from the Animal Services Division, John Curtin School of Medical Research (Australian National University, Canberra, Australia). Both KO strains had been backcrossed to BALB/c for at least 10 generations. Mice were housed in the Alfred Medical Research and Education Precinct animal facility. All experimental protocols were approved by the precinct Animal Ethics Committee.

Induction of Allergic Airway Inflammation and IL-13 Instillation

Mice were sensitized by intraperitoneal injection with ovalbumin (OVA) (50 µg/200 µl; Sigma-Aldrich, NSW, Australia) adsorbed to aluminum hydroxide gel (alum) (Reheis, Berkely Heights, NJ); control mice received 200 µl of saline mixed with alum. Ten days after sensitization, mice were challenged by intranasal instillation of 25 µg OVA in 50 µl saline on three consecutive days and killed 24 hours after the final challenge; control mice received saline alone. In certain experiments, mice were anesthetized with ketamine (40 mg/kg) (Parnell Laboratories, Alexandria, NSW, Australia) and xylazine (8 mg/kg) (Troy Laboratories, Smithfield, NSW, Australia) and challenged intratracheally every second day on four occasions (20). Intratracheal intubation was performed with a 20-gauge catheter attached to a 50-µl microsyringe (SGE International Pty. Ltd., Melbourne, Australia) and a blunted 20-gauge needle. Recombinant IL-13 (1.3 µg in 50 µl PBS/0.1% BSA; a gift from Genetics Institute, Cambridge, MA) was instilled intratracheally. In some experiments, IL-13 purchased from eBioscience (8.6 µg) (#34-8131; eBioscience, San Diego, CA) was used, with identical results. Control mice received saline or PBS in 0.1% BSA.

Bronchoalveolar Lavage, Differential Counts, and Tissue Sampling

Methods were as described previously (20). Blood was collected from the inferior vena cava, and serum was collected by centrifugation. BAL was performed with 0.4 ml 1% FCS in PBS followed by three lavages of 0.3 ml. Viable lung-draining LN cells and BAL leukocytes were counted in a hemocytometer. For differentials, BAL cytoposits were stained with Giemsa (Merck, Kilsyth, Victoria, Australia), and 200 cells were identified by morphologic criteria. Lungs were paraffin embedded, and 3-µm sections were stained with H&E or periodic acid-Schiff (PAS) for goblet cell quantitation.

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, or IL-13 antibody and blocked with 10% FCS in RPMI 1640 (Invitrogen, Mt. Waverly, Victoria, Australia). Cells (0.5×10^6) resuspended in 100 µl medium were added to the wells with 10 µl of OVA (final concentration, 25 µg/ml) or medium alone. Plates were incubated for 16 hours at 37°C. Cytokine detection was with biotinylated IL-4, IL-5, or IL-13 antibodies 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 (Autoimmune Diagnostika, Strassberg, Germany). Antibodies were from BD Pharmingen (San Jose, CA) or R&D Systems.

Activin and Follistatin Immunohistochemistry

Paraffin sections were dewaxed and rehydrated, and antigens were retrieved by immersing slides in 0.01 M citrate buffer (pH 6.0), heating in a 1,000W microwave oven (high for 2.5 min, low for 5 min), cooling at 4°C for 20 minutes, and washing in water for 5 minutes. Endogenous peroxidase was blocked in 3% H₂O₂ for 10 minutes, 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 antibodies specific for the activin β A

subunit (E4) or follistatin (2E6) at 10 mg/ml overnight at 4°C. 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 H₂O. 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).

OVA-Specific IgE ELISA

OVA-specific IgE was detected as described previously (20). Briefly, ELISA plates were coated with OVA (10 µg/ml) 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 ELISA and Follistatin Radioimmunoassay

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, 21). Follistatin concentrations were measured in serum and BAL fluid using a discontinuous radioimmunoassay as described previously (21). Note that 1 nM activin A = 25 ng/ml, and 1 nM follistatin = 35–45 ng/ml (range due to variable glycosylation and different isoforms). The activin A antibody (E4) used for the ELISA and immunohistochemistry was obtained from Professor Nigel Groome (Oxford Brookes University, United Kingdom). The follistatin antibodies (#204 and 2E6) used for the immunoassay and immunohistochemistry were developed in house and were raised against purified native bovine follistatin or human recombinant follistatin. The assay buffer for the BAL fluid samples was 0.05% BSA/PBS. The limit of activin A detection for serum and BAL fluid samples was 0.01 ng/ml. The limits of follistatin detection were 0.5 ng/ml and 1.04 ng/ml for serum and BAL fluid samples, respectively.

TGF- β ELISA

TGF- β was detected using a TGF- β ELISA kit according to the manufacturer's instructions (#DY1679; R&D Systems). Briefly, Costar ELISA plates (Corning Inc., Corning, NY) were coated with TGF- β 1 antibody and blocked (5% Tween 20 in PBS with 0.05% NaN₃). Latent TGF- β 1 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 to 7.6. Activated samples and serially diluted TGF- β standards were added and incubated for 2 hours. After samples were washed, biotinylated TGF- β -specific antibody was added for 2 hours. Detection was with streptavidin-horseradish peroxidase (R&D Systems), and development was with *o*-phenylenediamine (P5412; Sigma-Aldrich). Absorbance was read at 490 nm. The limit of TGF- β detection was 8 pg/ml.

IL-13 ELISA

IL-13 was detected in BAL fluid samples using the IL-13 ELISA Ready-SET-Go! kit (#88-7137; eBioscience). Fifty-microliter standards and sample were tested. Details were otherwise as specified in the manufacturer's instructions. Wavelength subtraction was used, with subtraction of 570 nm values from 450 nm values. The limit of detection was 3.9 pg/ml.

Normal Human Bronchial Epithelial Cell Culture

Cryopreserved normal bronchial epithelial cells (Clonetics Corp., San Diego, CA) were a generous gift from Dr. Ling Zheng (Department of Medicine, Monash University, Australia). The primary cells were passaged twice before freezing in liquid nitrogen. Cells were grown in small airway culture medium (Clonetics Corp.) supplemented with bovine pituitary extract (30 µg/ml), hydrocortisone (0.5 µg/ml), human recombinant epidermal growth factor (0.5 ng/ml), epinephrine (0.5 µg/ml), transferrin (10 µg/ml), insulin (5 µg/ml), retinoic acid (0.1 ng/ml),

triiodothyronine (6.5 ng/ml), gentamycin (50 µg/ml), amphotericin-B (50 ng/ml), and fatty acid-free BSA (0.5 mg/ml) (all from Clonetics Corp.). Cells were seeded at 18,000 cells per well on a 24-well culture plate (Costar) and incubated at 37°C in 5% CO₂, with 1.5 ml of medium added and changed every 48 hours. When cells were 80% confluent, recombinant human IL-13 or recombinant activin A (both from R&D Systems; reconstituted in 0.1% BSA/PBS) were added to final concentrations of 1, 10, or 100 ng/ml. Cell culture supernatants were collected at various time points for analysis.

Quantitative Image Analysis

Images of tissue sections were captured using a 20× objective and appropriately calibrated Image-Pro Plus 5.0 software (Media Cybernetics, Inc., Bethesda, MD). Bronchiole linear basement membrane length was measured (mm), and the number of activin A- or PAS-positive cells in the airway was counted, yielding the number of positive cells per millimeter of basement membrane. Ten airways per mouse were assessed.

Isolation of Total Lung RNA

Total RNA was extracted from the left lung lobe of experimental animals by homogenizing the tissue in TRIzol Reagent (Invitrogen, Carlsbad, CA). After chloroform extraction, the RNA was precipitated with isopropanol and washed with 70% EtOH before being redissolved in water.

cDNA Synthesis and Quantitative Real-Time PCR

Quantitative real-time PCR was performed using cDNA generated by reverse transcription of the total RNA using random hexamers and M-MLV Reverse Transcriptase (Promega, Madison, WI). Gene-specific oligonucleotide primer pairs were designed using the web-based Primer3 software, with at least one of the primers from each pair overlapping an exon-exon boundary to eliminate amplification of genomic DNA. The reaction was performed in triplicate for each cDNA sample as a normalizing control, and levels of 18S rRNA were determined using 18S-specific primers. Each reaction consisted of 10 µl SYBR GreenER qPCR SuperMix Universal (Invitrogen), 1 µl primer mix (10 µM each of forward and reverse), 4 µl water, and 5 µl cDNA template of suitable concentration. To minimize operator-introduced pipetting errors, all reactions were prepared using a CAS-1200 pipetting robot (Corbett Research, Sydney, NSW, Australia). The thermal cycling and real-time PCR product detection was performed on a Rotor-Gene 3000 (Corbett Research). Data were analyzed using RotorGene 6000 software v1.7, and

differential expression was determined using the comparative delta delta CT method (22).

Primer Sequences

The primer sequences were as follows. *Acvr1*, forward: AGACAG CACTCTAGCGGAAGT; reverse: CTGTACCAGGAAAGGAA GACC. *Acvr1b*, forward: TTCTGGAGGAGTCCATGAAG; reverse: ACAACCTTTCGCATCTCCT. *Acvr2a*, forward: CAGATGGACCC GTAGATGAG; reverse: GCACAACAACCTTCTGCATA. *Acvr2b*, forward: TACGACAGGCAGGAGTGTG; reverse: CGCAGCAG CAGAAGTACAC. *Inhba*, forward: TCACCTTTGCCGAGTACAG; reverse: GAAGAGCCCACTTCTGCAC.

Statistical Analysis

Statistics were analyzed with GraphPad Prism v5.02 software. Data were analyzed for normality and log transformed as necessary before analysis by ANOVA or two-way ANOVA with Bonferroni post tests. Correlations were assessed with Spearman analysis. Differences were considered statistically significant at $P \leq 0.05$. Group sizes are indicated in the figure legends. All values are mean $\pm$ SEM.

RESULTS

IL-13 Induces Activin A Secretion into BAL Fluid in the Naive Mouse Lung

Intratracheal instillation of IL-13 into the lungs of naive mice significantly increased lung-draining LN cell and BAL eosinophil numbers at Day 7 (Figures 1A and 1B). Eosinophil frequency at Day 7 was $20.4 \pm 5.4\%$ versus $0.5 \pm 0.5\%$, IL-13 versus saline, respectively. IL-13 instillation caused significant increases in BAL fluid activin A concentrations at Day 3 and Day 7 (Day 3, 6.5-fold; Day 7, 11-fold) (Figure 1C). Consistent with previous reports, IL-13 instillation also increased BAL fluid TGF- β concentrations with earlier kinetics than activin A, although the increase was of lesser magnitude, peaking at 4.7-fold at Day 3. Together, these data show that IL-13 induces eosinophilic airway inflammation and increased activin A and TGF- β secretion into BAL fluid.

IL-13 Does Not Increase Activin β A or Activin Receptor Expression

To investigate whether IL-13-induced increases in BAL fluid activin A were dependent on activin synthesis, we analyzed activin

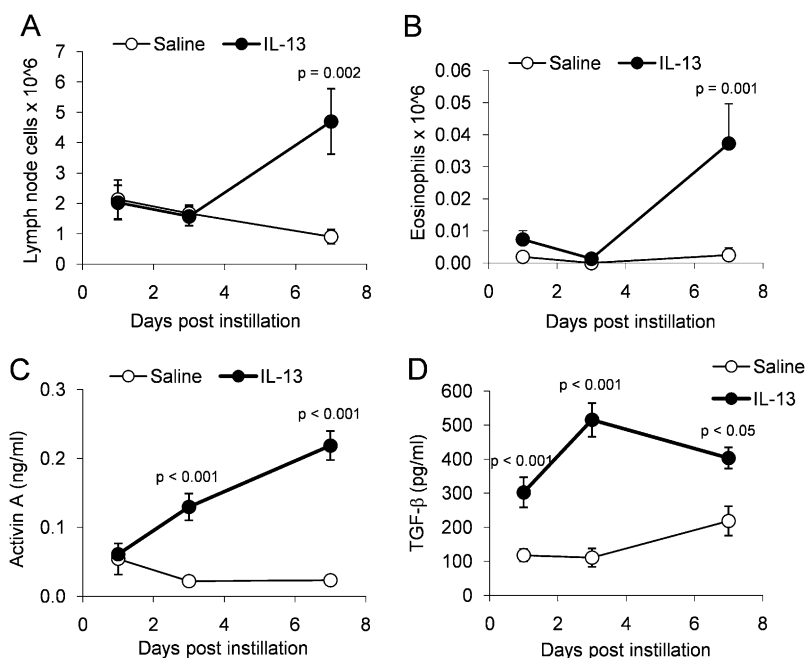


Figure 1. Exogenous IL-13 induces pulmonary inflammation and increases bronchoalveolar lavage (BAL) fluid activin A and TGF- β concentrations in naive mice. IL-13 was instilled intratracheally into naive BALB/c mice on Day 0. Control mice received saline. (A) Lung-draining lymph node (LN) cell numbers. (B) Absolute BAL eosinophils as determined by differential analysis. (C) BAL fluid activin A concentrations. (D) BAL fluid TGF- β concentrations. P values are for IL-13 versus saline groups at a given time point. Data are mean $\pm$ SEM. $n = 5$ to 7 mice per group per time point.

β A mRNA expression. Quantitative real-time PCR on whole lung RNA showed that activin β A expression was not increased at any time point. PBS instillation induced a transient but nonsignificant increase in activin β A mRNA at Day 3 (see Figure E1 in the online supplement). Similarly, IL-13 treatment of naive mice did not increase activin receptor expression. However, relative to PBS-treated control mice, IL-13-treated mice had significantly decreased expression of activin A type I receptors ActR-I and ActR-IB at Day 3 (ALK-2 and ALK-4, respectively), with no change at Days 1 and 7 (Figure E2). There were no significant changes in activin type II receptor expression at any time point. These data suggest that activin A secretion into BAL is dependent on preformed activin A.

IL-13 Induces Activin A Secretion from Human Bronchial Epithelium

To test whether IL-13 induces activin A secretion by airway epithelium, we cultured human small bronchial epithelial cells and exposed them to different concentrations of IL-13. IL-13 induced a dose-dependent secretion of activin A from airway epithelial cells, with a modest but significant effect at 10 ng/ml, increasing at 100 ng/ml (Figure 2). A kinetic analysis showed a marked increase in activin A concentrations in culture supernatants within 3 hours after addition of IL-13, and this concentration did not increase further at 24 hours. In a separate experiment, IL-13 (100 ng/ml) increased activin A concentrations in culture supernatant at 24 hours but not at 48 hours, suggesting that the increased activin A concentrations at 24 hours or less are due to secretion of prestored protein and that *de novo* activin A synthesis and secretion do not occur to any measurable degree (Figure 2; data not shown). Collectively, these data show that airway epithelial cells are a major source of activin A and suggest that activin A is prestored and available for rapid secretion.

IL-13 Drives Loss of Activin A Immunoreactivity by Airway Epithelium, Mucus Cell Metaplasia, and Increased Activin A Secretion

IL-13 is a key cytokine driving goblet cell metaplasia (14–16). Our previous studies showed concomitant increased airway BAL fluid activin A concentration, loss of airway epithelial activin A localization, and increased frequency of mucus-producing cells (3), suggesting a relationship between IL-13, activin A secretion, and mucus cell metaplasia. We therefore determined the frequency of mucus-producing and activin A-positive airway epithelial cells in IL-13-treated and control mice, identified by PAS staining and activin A immunohistochemistry, respectively (Figures 3A–3D). IL-13 instillation markedly increased the frequency of PAS-positive cells at Day 3 and Day 7 (Figures 3C and 3E) and caused a small but significant decrease in the frequency of activin A-immunopositive epithelial cells (Figures 3D and 3F). These data show that IL-13 is a common driver of loss of activin A immunoreactivity and mucus hypersecretion by airway epithelium. We also show in an acute asthma model that increased endogenous IL-13 production correlates with significant decreases in activin A immunopositive airway epithelial cells and a concomitant significant increase in BAL fluid activin A (Figure E3). BAL fluid IL-13 concentrations peaked after three allergen challenges at 190 pg/ml (Figure E4A), returning to baseline by 2 to 4 days after the final challenge. The increased IL-13 concentrations immediately preceded the peak in activin A concentrations (3), reinforcing the idea that IL-13 drives activin A secretion *in vivo*. In contrast, BAL fluid IL-13 concentrations in IL-13-treated mice peaked at 600 pg/ml at Day 1, returning to baseline by Day 7 (Figure E4B).

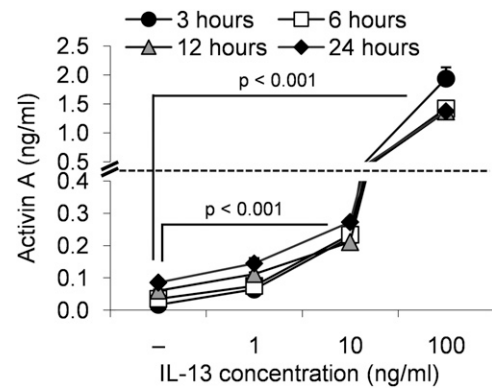


Figure 2. Exogenous IL-13 induces activin A production by human bronchial epithelial cells. Normal human small airway epithelium was grown until 80% confluent. Human IL-13 was added at various concentrations, and culture supernatant was analyzed at the indicated time points. *P* values for a given IL-13 concentration apply to each time point. Triplicate samples, mean $\pm$ SEM. One of two similar experiments is shown.

Increased BAL Fluid Activin A during Allergic Airway Inflammation Is IL-13 Dependent

We examined the IL-13 dependence of activin A secretion during acute allergic airway inflammation in IL-13-deficient mice (IL-13 KO) mice. Wild-type (WT) and IL-13 KO mice were sensitized and challenged with OVA (Figure E5A). As expected, there was a significant increase in total BAL cell counts in OVA-sensitized/challenged WT mice (WT/OVA), compared with WT/saline control mice, associated with a 35-fold increase in eosinophil numbers (Figure E5B). In contrast, compared with WT/OVA mice, total BAL cell and eosinophil numbers were decreased (3- and 6-fold, respectively) in IL-13 KO/OVA mice. IL-13 KO/OVA mice had 3-fold lower serum OVA-specific IgE concentrations than WT/OVA mice (Figure E5C). These results are consistent with the role for IL-13 in promoting airway eosinophilia and IgE synthesis (15, 23, 24). Specifically, examination of allergen-induced Th2 cytokine production in these mice showed that IL-13 KO/OVA mice had a 7-fold decreased frequency of IL-4-producing cells and a 2-fold decreased frequency of IL-5-producing cells compared with WT/OVA control mice (Figures 4A and 4B). The frequency of IL-13-producing cells in IL-13 KO/OVA mice was undetectable, whereas there was a significant up-regulation of these cells in WT/OVA mice compared with the IL-13 KO/OVA or WT/saline groups (Figure 4C). These results show that IL-13 deficiency decreases the frequency of IL-4- and IL-5-producing cells in the lung-draining LN, consistent with an important role for IL-13 in Th2 cytokine production (23). Overall, IL-13 KO/OVA mice had greatly reduced severity of airway inflammation when compared with WT/OVA mice, with lungs resembling WT/saline controls (Figure E6). WT/OVA mice had a high frequency of goblet cells, whereas these cells were absent in IL-13 KO/OVA mice, consistent with the critical role of IL-13 in driving airway mucus production (14–16) (Figure E6).

Next, we determined the effect of IL-13 deficiency on activin A release. As we showed previously (3), BAL fluid activin A concentrations increased 5-fold in WT/OVA mice. However, relative to WT/OVA mice, BAL fluid activin A concentrations in IL-13 KO/OVA mice were 4-fold lower (Figure 5A). Activin A concentrations in IL-13 KO/saline mice were significantly decreased compared with WT/saline mice, suggesting a role for IL-13 in regulating basal activin A concentrations in BAL fluid. Concentrations of the activin binding protein follistatin were

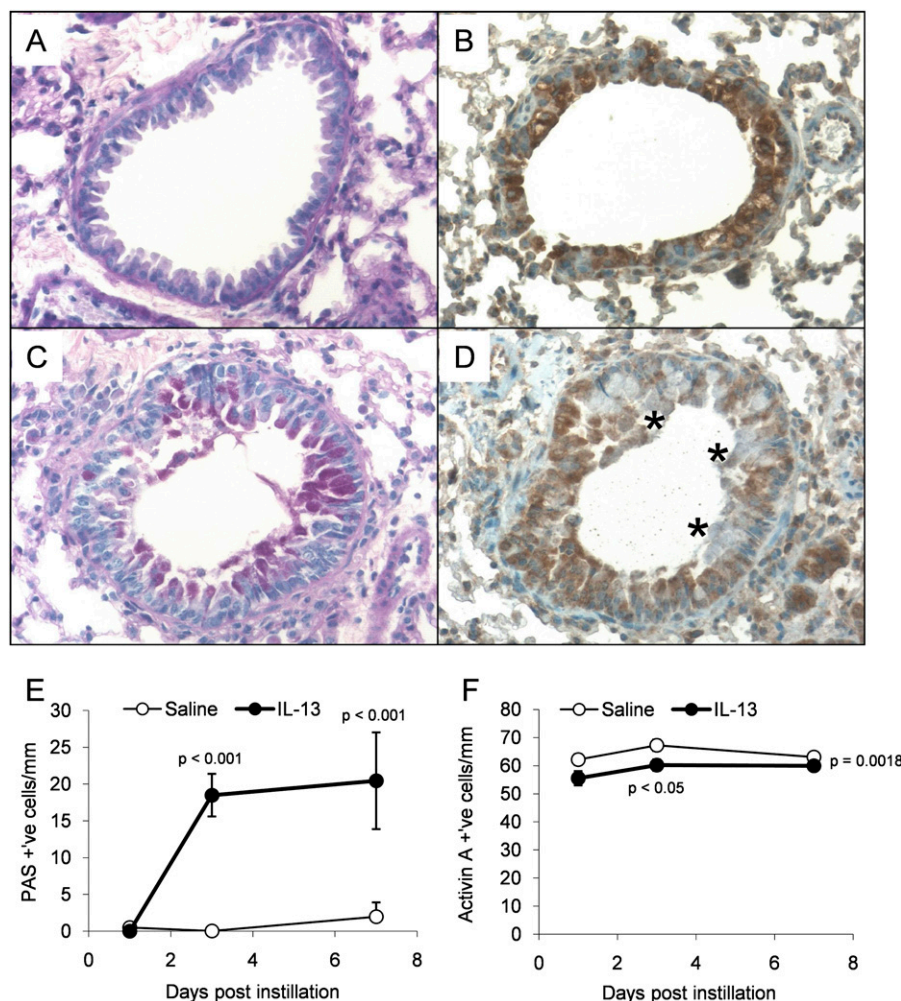


Figure 3. Exogenous IL-13 induces mucus production and loss of activin A immunostaining by airway epithelium in naive mice. IL-13 was instilled intratracheally into naive BALB/c mice on Day 0. Control mice received saline. Representative lung tissue sections showing Periodic acid-Schiff staining (A, C) and activin A immunohistochemistry (B, D) from saline-treated mice (A, B) and IL-13-treated mice (C, D). (D) *Loss of activin A localization. Frequency of (E) airway Periodic acid-Schiff-positive cells and (F) activin A-immunoreactive cells per millimeter basement membrane as determined by quantitative image analysis. (A–D) Original magnification: $\times 400$. Representative sections are shown. (F) *P* values are for IL-13 versus saline groups at a given time point or between treatment groups. Data are mean $\pm$ SEM. *n* = 5 to 7 mice per group per time point.

significantly increased in BAL fluid of WT/OVA mice compared with WT/saline control mice, whereas the concentrations in IL-13 KO/OVA mice were significantly lower than WT/OVA mice (Figure 5B), consistent with a role for activin driving follistatin release *in vivo* (10). BAL fluid TGF- β concentrations in WT/OVA mice were increased relative to WT/saline control mice, and TGF- β concentrations in IL-13/OVA mice were slightly decreased relative to WT/OVA mice, although these changes were not statistically significant (Figure 5C). These data confirm that IL-13 is an important driver of activin A extracellular release in acute allergic airway inflammation and suggest partial regulation of follistatin and TGF- β by IL-13 or activin A.

Eosinophils Are Not a Major Source of Activin A in Mice with Allergic Airway Inflammation

Eosinophils have been postulated to be a major source of TGF- β in experimental asthma (25, 26), although this remains controversial (27). Given the overlaps in TGF- β and activin signaling, we postulated that eosinophils may also be a source of activin A during allergic airway inflammation. To test this hypothesis, we instilled IL-13 into naive mice and IL-5/eotaxin DKO mice, which lack eosinophils (28). IL-13 induced increases in draining LN cell numbers in WT mice, and this was only slightly attenuated in IL-5/eotaxin DKO mice (Figure 6A). The absolute number of BAL eosinophils in WT mice increased 30-fold 7 days after mice were given IL-13, consistent with a crucial role for IL-13 in the accumulation of airway and intraepithelial eosinophils, whereas airway eosinophilia was

absent in IL-5/eotaxin DKO mice, as expected (Figure 6B). Although IL-13 instillation increased BAL fluid activin A concentrations 15-fold in WT mice, a similar increase was observed in IL-5/eotaxin DKO mice (Figure 6C). IL-13 instillation also increased TGF- β concentrations in WT mice, with a similar increase observed in IL-5/eotaxin DKO mice (Figure 6D). Collectively, these findings show that eosinophils are not a major source of IL-13-induced increases in airway activin A and TGF- β .

DISCUSSION

Our data demonstrate that IL-13 plays a key role in the regulation of activin A secretion from airway epithelium. When instilled into the trachea of naive mice, IL-13 caused increased BAL fluid concentrations of activin A. Furthermore, IL-13 caused a dose-dependent increase in activin A secretion from cultured normal human airway epithelium, indicating that airway epithelium is a major source of activin A. We confirmed an important role for IL-13 in driving increased BAL fluid activin A concentrations by comparing allergic airway inflammation in WT and IL-13 KO mice. Finally, our data show that IL-13 induced equivalent increases in BAL fluid activin A concentrations in WT and eosinophil-deficient IL-5/eotaxin DKO mice, indicating that eosinophils are not a major source of activin A. Our data define an important role for IL-13 in the regulation of activin A secretion by bronchial epithelial cells in naive mice and in allergic airway inflammation.

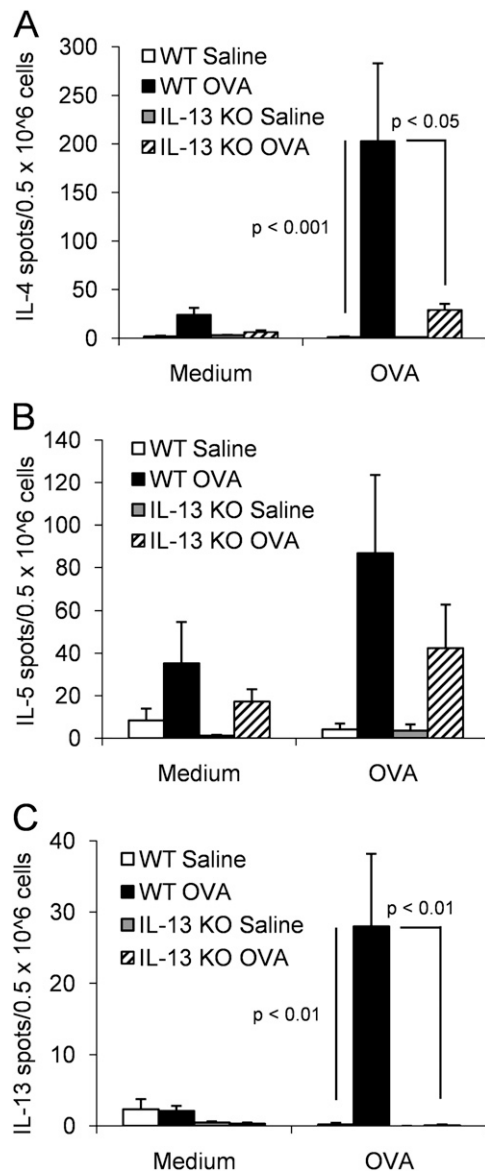


Figure 4. IL-13 deficiency decreases the frequency of Th2 cytokine producing cells in the lung-draining LNs during experimental asthma. Mice were sensitized and challenged with ovalbumin (OVA) as described in Figure E5A. Lung draining LN cells were stimulated with medium or OVA. Frequency of (A) IL-4-, (B) IL-5-, and (C) IL-13-producing cells was measured by ELISPOT. Each condition was analyzed in triplicate. Data are mean $\pm$ SEM. $n = 7$ mice per group.

We previously showed that the strong activin A localization in airway epithelium in normal mice is lost during the course of allergic airway inflammation, concomitant with increased BAL fluid activin A concentrations (3). The presence of activin A in normal airway epithelium and its loss during allergic inflammation is similar to the changes seen for the closely related cytokine TGF- β in patients with asthma (29, 30) and in experimental models of acute and chronic allergic airway inflammation (31, 32). The increased BAL fluid activin A concentrations were related to the magnitude of the Th2 inflammatory response, suggesting that Th2 cytokines regulate activin A secretion. The possibility that IL-13 regulates activin A localization in allergic airway inflammation (3) is supported by our data showing that (1) IL-13 potentially induces secretion of activin A from airway epithelium *in vitro*, (2) IL-13 increases BAL fluid activin A

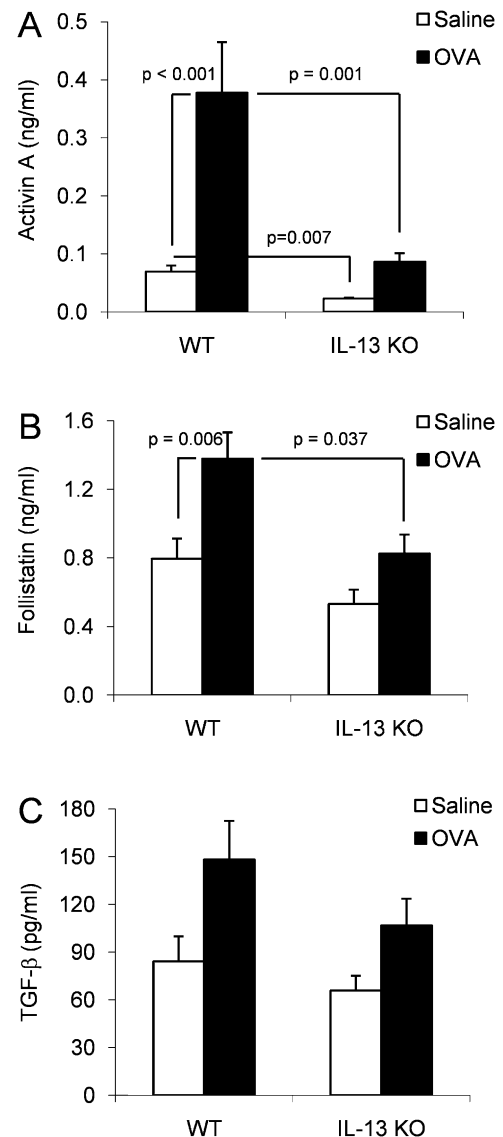


Figure 5. IL-13 deficiency decreases BAL fluid activin A, follistatin, and TGF- β concentrations during experimental asthma. Mice were sensitized and challenged with OVA as described in Figure E5A. BAL fluid was analyzed for (A) activin A, (B) follistatin, and (C) TGF- β by ELISA (A and C) and radioimmunoassay (B). Data are mean $\pm$ SEM. $n = 7$ mice per group.

concentrations in naive mice, and (3) the increase in BAL fluid activin A concentrations that occurs in experimental asthma is decreased in IL-13 KO mice. The finding of decreased follistatin concentrations in IL-13 KO mice is consistent with the idea that activin A drives follistatin release *in vivo* (10). Other researchers have shown that Th2 cytokines induce activin A secretion from cultured human fibroblasts (4). However, activin A was not detected in submucosal fibroblasts in naive mice despite strong epithelial staining (3, 4), suggesting that fibroblasts are a less important source or store of activin A than airway epithelium. Similarly, IL-4 and IL-13 induce a dose-dependent increase in TGF- β secretion from cultured human bronchial epithelium but not human fetal lung fibroblasts (17). The possibility that other cell types, such as macrophages and dendritic cells (DCs), contribute to the increased activin A concentration in BAL fluid during allergic airway inflammation is not excluded. This contention is supported by the recent demonstration that activated

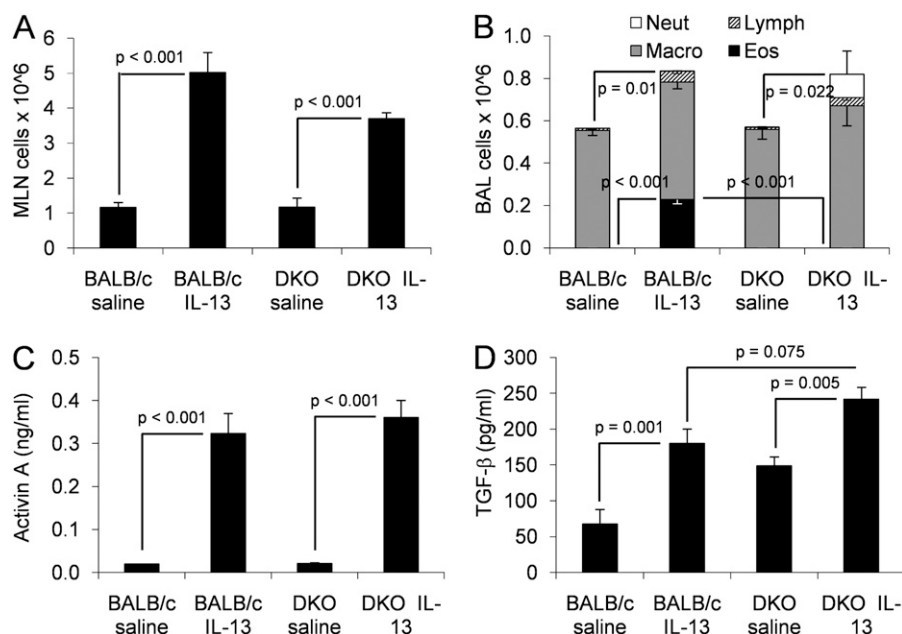


Figure 6. Increased BAL fluid activin A and TGF- β concentrations in IL-13-treated mice are not dependent on eosinophils. Saline or IL-13 was instilled intratracheally into BALB/c WT or eosinophil-deficient IL-5/eotaxin DKO mice and analysis performed 7 days later. (A) Lung-draining LN cell counts. (B) BAL differential analysis showing absolute cell counts. (C) BAL fluid activin A concentrations. (D) BAL fluid TGF- β concentrations. Data are mean $\pm$ SEM. $n = 7$ to 8 mice per group.

DCs can produce large amounts of activin A, at least *in vitro* (33). However, as we and others have shown, IL-13 KO mice have diminished IL-4 and IL-5 production (23), and we do not exclude the possibility that these Th2 cytokines also regulate activin A compartmentalization.

Our previous study in a model of acute allergic airway inflammation showed that blocking activin A with follistatin decreases mucus production in the lung, suggesting that activin A also regulates IL-13 production (3). However, additional studies by our group showed no evidence for the direct effects of activin A on mucus production in naive mouse lungs (data not shown), consistent with an indirect effect of activin A on mucus production via IL-13. The fact that follistatin instillation immediately before allergen challenge decreases Th2 cytokines and mucus production suggests that activin A is important during the challenge phase. However, we show here that IL-13 KO mice have decreased basal activin A concentrations (i.e., at the time of allergen sensitization) and markedly reduced severity of allergic airway inflammation. These latter findings raise the possibility that activin A also plays a role in the sensitization phase. Because there is no activin A bioassay suitable for use with serum or BAL, we cannot say that the decreased activin A concentrations in IL-13 KO mice equate to decreased activin A bioactivity. The decreased airway inflammation in these mice, however, indirectly supports this idea.

We reported previously that the loss of activin A immunoreactivity from inflamed airway epithelium is mirrored by an increased goblet cell frequency (3), suggesting that metaplasia to a mucus-producing phenotype is linked to increased epithelial cell secretion of activin A. In this study, we showed that the frequency of activin A-positive cells showed a small but significant decrease upon IL-13 instillation, whereas there was a marked increase in the frequency of mucus-producing cells, suggesting a weak relationship between these events. However, in our murine model of allergic airway inflammation, the maximal BAL fluid activin A concentration was approximately 1.2 ng/ml (3), compared with 0.22 ng/ml after IL-13 instillation. Conversely, the frequency of goblet cells in allergic airway inflammation was 64 per millimeter of basement membrane, compared with 20 per millimeter of basement membrane after IL-13 instillation. These findings show that the magnitude of BAL

fluid activin A concentrations (and the concomitant loss of epithelial immunoreactivity) reflect the extent of mucus metaplasia. Thus, exogenous IL-13 induces these changes less potently than acute allergic airway inflammation. Together, these observations suggest that the local IL-13 tissue concentration critically regulates activin A epithelial expression and the extent of goblet cell metaplasia. The possibility exists of a direct mechanistic link between mucus metaplasia and loss of epithelial activin A immunoreactivity, although our data do not address this directly.

Activin A is a homodimer of activin β A subunits. Given that IL-13 increased activin A secretion, we questioned whether this was reflected in increased activin β A subunit expression. Our data show that IL-13 did not increase expression of activin β A or activin type I or II receptors. PBS instillation caused a transient increase in activin type I receptor expression (ActR-1 and ActR-1B) at Day 3. The process of intratracheal intubation causes very mild transient inflammation (unpublished data), and it is possible that such inflammation results in the up-regulation of other TGF- β superfamily ligands (e.g., inhibin, nodal, bone morphogenetic protein, and growth and differentiation factor) that also use these activin receptors (34). Our data show that induction of activin A by IL-13 is not associated with increased activin β A transcription. Furthermore, in our allergic asthma model, the loss of activin A immunoreactivity from airway epithelium and the concomitant increase in BAL fluid activin A concentrations correlate with increased IL-13 production *in vivo*. Together with the findings that IL-13 induces identical changes to activin A compartmentalization in naive mice, these data therefore suggest that activin A is prestored in airway epithelium and progressively secreted during the allergic inflammatory response and that IL-13 is an important driver of this process.

Studies with IL-5 transgenic mice have shown that eosinophils produce TGF- β and that BAL fluid TGF- β concentrations correlate with airway eosinophil numbers (25, 26), suggesting that eosinophils are a significant source of TGF- β . However, TGF- β concentrations were not dramatically lower in chronically challenged, eosinophil-deficient mice (27). In the present study, IL-13 intratracheal instillation increased activin A BAL fluid concentrations by Day 3, whereas eosinophil numbers increased significantly at Day 7 (see Figure 1), suggesting that

eosinophils are not primarily responsible for the increased activin A concentrations. Given the ambiguity regarding the eosinophil as a source of TGF- β and the overlaps in biology of TGF- β and activin A, we sought to more rigorously determine whether eosinophils contribute to the IL-13-induced activin A secretion using IL-5/eotaxin DKO mice that have abrogated airway and tissue eosinophilia (28). As expected from our previous finding, IL-13 caused increased BAL fluid activin A and TGF- β concentrations in WT mice. However, these values were not significantly different from IL-5/eotaxin DKO mice. Eosinophils are therefore not a major contributor to the IL-13-induced increase in BAL fluid activin A concentrations.

Circulating concentrations of activin A increase very early in LPS-induced systemic inflammation before IL-6 and TNF (10, 11). Exogenous IL-13 induced a very rapid (within 3 h) secretion of activin A *in vitro*, whereas *in vivo* significantly increased activin A BAL fluid concentrations were not detectable until Day 3. Consistent with these *in vivo* findings, BAL fluid activin A concentrations are increased after three to four allergen challenges in an acute asthma model (3). The different kinetics may be due to differences between isolated cultured airway epithelial cells and the complex *in vivo* micro-environment. Factors such as the local cytokine milieu, antigen-presenting cell (such as DCs) functional status, and relative IL-13 concentration are likely influences on the kinetics of activin A secretion.

Collectively, our findings show that in addition to the known important roles for IL-13 in the development of key asthma symptoms, IL-13 induces activin A secretion from airway epithelium. Our findings indicate a possible relationship between the degree of mucus metaplasia and the extent of increased BAL fluid activin A concentrations, likely reflecting local tissue IL-13 concentrations. Our findings also suggest that eosinophils are not a major contributor to the increased airway activin A concentrations. Because activin A has pro- and antiinflammatory properties (10, 11, 33, 35), induction of activin A release during allergic airway inflammation may drive or dampen the allergic inflammatory response depending on dose or timing. Our data show that activin A secretion is further regulated by the Th2 cytokine IL-13, indicating a complex regulatory circuit. It is possible that during chronic inflammation in the lung, sustained activin A production contributes to airway remodeling directly or via induction of TGF- β . Together, these findings underscore the idea that, along with TGF- β , activin A plays an important role in asthma pathogenesis and suggest that targeting this pathway holds therapeutic potential.

Conflict of Interest Statement: D.P. received a U.S. patent for a method for inhibiting activin molecular suppression. He and his institution have received no financial benefit. None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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