

Activin A is an acute allergen-responsive cytokine and provides a link to TGF- β -mediated airway remodeling in asthma

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Background: Allergic asthma typically shows activated, allergen-specific CD4⁺ T cells in the early phase and airway remodeling in the late phase of the disease. Although TGF- β plays a crucial role in airway remodeling, it is only marginally induced in CD4⁺ T cells in the early allergen-dependent activation of the immune system.

Objective: To elucidate the transition between early- and late-phase events, we investigated the role of activin A, a close family member of TGF- β .

Methods: Activin A and TGF- β ₁ levels were measured systemically in the serum and in CD4⁺ T cells of asthmatic patients, as well as locally in the lung.

Results: Activin A serum levels were increased in patients with severe asthma compared with levels in patients with moderate asthma and healthy control subjects, whereas all patients showed significantly increased TGF- β ₁ serum levels independent of disease severity. In T cells only patients with moderate asthma showed increased activin A mRNA expression, whereas TGF- β ₁ expression was equal to that seen in healthy subjects. Accordingly, ovalbumin sensitization in a mouse model of allergic asthma could induce activin A mRNA expression, but not TGF- β ₁ expression, in the lung. Immunohistochemistry of mice and human specimens revealed an abundant expression of activin A by infiltrating lymphocytes and structural cells of the lung. Although TGF- β ₁ more potently enhanced proliferation and Smad 2/3-dependent reporter genes in fibroblasts, activin A was capable of inducing TGF- β ₁ and vice versa.

Conclusion: Activin A provides a link between acute allergen-specific T-cell responses and chronic TGF- β ₁-mediated airway remodeling in asthma. (*J Allergy Clin Immunol* 2006;117:111-8.)

Key words: T cells, fibrosis, tolerance, suppression, allergy

Asthma is a chronic inflammatory disease of the airways in which activated CD4⁺ T cells play a key role. One of the major cytokines involved in the pathogenesis of asthma is TGF- β , which is produced by T cells and structural cells of the lung.¹⁻³ TGF- β is a well-known suppressive cytokine that mediates peripheral tolerance toward allergens and autoantigens.⁴ It also induces extracellular matrix deposition and thus contributes critically to fibrosis in the airways and lungs.⁵⁻⁷ These functions of TGF- β , which occur in later stages of inflammation, prevent lymphocytes from activation and assist during wound repair. In the chronically inflamed lung prolonged activation and expression of TGF- β as a continuous attempt to repair inflammation-induced tissue destruction leads to an irreversible remodeling of the airways.^{6,8} Currently, the link between allergen-specific immune responses and TGF- β -induced remodeling is unknown because allergen challenge increases TGF- β levels in the lung but not in activated T cells.^{1,9-11} Furthermore, immunohistochemical analysis revealed an abundant TGF- β expression in the asthmatic airway epithelium, along with intracellular activated Smad 2/3 and increased TGF- β levels in the bronchoalveolar lavage fluid,¹ whereas healthy airways did not show such activation patterns.^{12,13} These findings are also confirmed by measurements of TGF- β in bronchoalveolar lavage fluids after segmental allergen challenge in asthmatic subjects and in a murine model of allergic asthma.^{1,14} Taken together, allergens clearly lead to TGF- β expression in structural cells of the lung in human subjects and mice.

Closely related to TGF- β is the cytokine activin A, which is not yet as well investigated as TGF- β but that appears to be structurally and functionally similar to TGF- β . It was initially purified as a gonadal protein that releases a follicle-stimulating hormone from the anterior pituitary gland.^{15,16} Interestingly, activin A was shown to respond immediately to stimuli such as LPS *in vivo*,¹⁷ whereas TGF- β does not participate in this acute and early response.^{18,19} Activin A is produced by many cells, including T cells, monocytes, macrophages, bone marrow cells, mast cells, and stromal cells.^{20,21} Although TGF- β and activin A share the same intracellular Smad signaling pathway, activin A binds to its own specific transmembrane serine-threonine kinase receptors.^{22,23} Activin A is a

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Abbreviations used

GC: Glucocorticoid
OVA: Ovalbumin
PMA: Phorbol myristate acetate

homodimer of 2 β A subgroups.¹⁵ A naturally occurring antagonist of activin A, inhibin α , also consists of 1 β A subunit but differs in an additional inhibin α subgroup. A second antagonist, follistatin, can directly bind activin A and thus neutralizes its biologic activity, whereas inhibin α exerts its antagonistic effects on the level of ligand biosynthesis, receptor binding, or signal transduction. Activin A has a variety of functions in several cell types. It has been described to induce apoptosis in cells that regulate growth and development in the early phases of embryogenesis and hemopoiesis.^{24,25} It also promotes the proliferation of fibroblasts in the human lung and affects the growth of smooth muscles of the human airway.²⁶ Like TGF- β , recent evidence suggests that activin A might also play an important role in the pathogenesis of asthma and lung fibrosis.²⁶⁻²⁸ In particular, human mast cells were described to be an abundant source of activin A secretion.²⁶ The current study focuses on the expression pattern and function of activin A and TGF- β in the pathogenesis of asthma and attempts to elucidate the relationship between allergen-specific responses and local TGF- β -mediated remodeling processes.

METHODS**Patients and healthy control subjects**

Heparinized blood was taken from healthy nonallergic subjects and asthmatic patients from the Hochgebirgsklinik Davos-Wolfgang. For the activin A and TGF- β ELISA measurement, 14 healthy subjects, 15 patients with moderate asthma without glucocorticoids (GCs), 20 patients with moderate asthma with GCs, and 29 patients with severe asthma were studied. The patients had either moderate or severe asthma (as defined by the American Thoracic Society and the European Respiratory Society,²⁹ <http://www.ginasthma.com>) and were treated according to the recent guidelines. These patients were classified as atopic or nonatopic on the basis of their medical history and skin prick test responses to animal dander, food allergens, pollens, molds, and latex. Patients included in the study had positive skin prick test responses and a corroborating medical history confirming allergen-induced asthma. Healthy donors were included on the basis of a negative history of allergy, asthma, atopic dermatitis, and infectious disease. The local ethics committee reviewed and approved the study.

Mouse model of allergic asthma

BALB/c mice were immunized intraperitoneally with 10 μ g of ovalbumin (OVA; grade V; Sigma Chemical Company, St Louis, Mo) in 200 μ L of PBS or PBS alone and on days 0 and 21 and 1 week later on days 28 and 29 underwent nebulization with 1% OVA in PBS or PBS alone by using an ultrasonic nebulizer for 60 minutes twice daily. For secondary memory responses, mice recovered from the acute response of allergic asthma from day 29 to day 257 without any inhalation. The recovery phase is characterized by the presence of OVA-specific IgG₁ (no OVA-specific IgE after 4 months) and persistent peribronchial lung infiltrates containing predominantly CD4⁺

T cells, B cells, and macrophages for the lifetime of the mice. Rechallenge with aerosol OVA induces a rapid upregulation of T_H2 cytokines, followed by eosinophilic lung inflammation, mucus hypersecretion, airway hyperresponsiveness, and increased serum titers of OVA-specific IgE and IgG₁. On days 257 and 258, the mice were treated with 1% OVA in PBS by using an ultrasonic nebulizer for 60 minutes twice daily. The control group was treated in the same way with PBS but without OVA. Forty-eight hours after the final aerosol challenge, on day 260, we perfused the lungs with PBS and fixed them with 4% formalin for immunohistochemistry. Mice were nebulized with 1% OVA for 1 hour twice daily for 2 consecutive days to analyze total RNA expression after aerosol challenges. Lungs were removed and flash frozen in liquid nitrogen at 2 and 12 hours after the second aerosol challenge on both days. RNA was isolated from frozen lungs with TriZol and was maintained at -80°C until further use. OVA-sensitized mice showed a typical asthma phenotype, as demonstrated elsewhere³⁰ and in Fig E1 in the Online Repository of this article at www.jacionline.org.

Immunohistochemical staining

The formalin-fixed and paraffin-embedded lung sections were incubated overnight at 55°C and then deparaffinized with xylene and rehydrated. Slides were heated to 90°C for 5 minutes in 10 mM sodium citrate buffer (pH 6.0). Cold slides were incubated in peroxidase block buffer of the Dako LSAB+ HRP System (DakoCytomation, Glostrup, Denmark) and washed with TRIS-buffered saline with Tween (TBST). Slides were preincubated with 25% FCS and 0.1% BSA in TBST and washed, and an anti-activin β A (AF338; RnD Systems, Abingdon, United Kingdom), which only recognizes the β A subunit, or rabbit IgG fraction (DakoCytomation) was added as an isotype control at a concentration of 1 μ g/mL in blocking buffer. All sections were counterstained with hematoxylin and analyzed with an Axiovert microscope and an AxioCam digital camera (both Zeiss, Göttingen, Germany). The corresponding isotype control revealed only a weak staining of smooth muscle cells, so that these cells could not have been analyzed accurately.

Additional details on cell-culture techniques, CD4⁺ T-cell isolation, quantitative real-time PCR with primer sequences, and ELISA measurements were done as described previously^{31,32} and are shown in an online data supplement (see this article's Table E1 and Methods in the Online Repository at www.jacionline.org).

Statistics

We used GraphPad prism 4.0 for Macintosh computers (GraphPad Software Inc, San Diego, Calif) to analyze our statistics and verified all data for Gauss distribution by using the D'Agostino and Pearson omnibus normality test. To calculate differences between groups, we used Student *t* tests, and data were logarithmized before any Student *t* tests were performed to calculate absolute values. We considered any difference with a *P* value of .05 or less to be statistically significant. Error bars in each figure indicate the SD of each experiment.

RESULTS**Increased serum levels of TGF- β 1 and activin A in asthmatic patients**

Patients with moderate asthma without GC treatment showed significantly increased serum levels of activin A (1.155 ± 0.427 ng/mL, *n* = 15) compared with those seen in healthy control subjects (0.141 ± 0.77 ng/mL, *n* = 13) or patients with moderate asthma (0.259 ± 0.106 ng/mL, *n* = 22, *P* $\leq$.05; Fig 1, A). No significant difference was observed in relation to patients with severe asthma ($0.680 \pm$

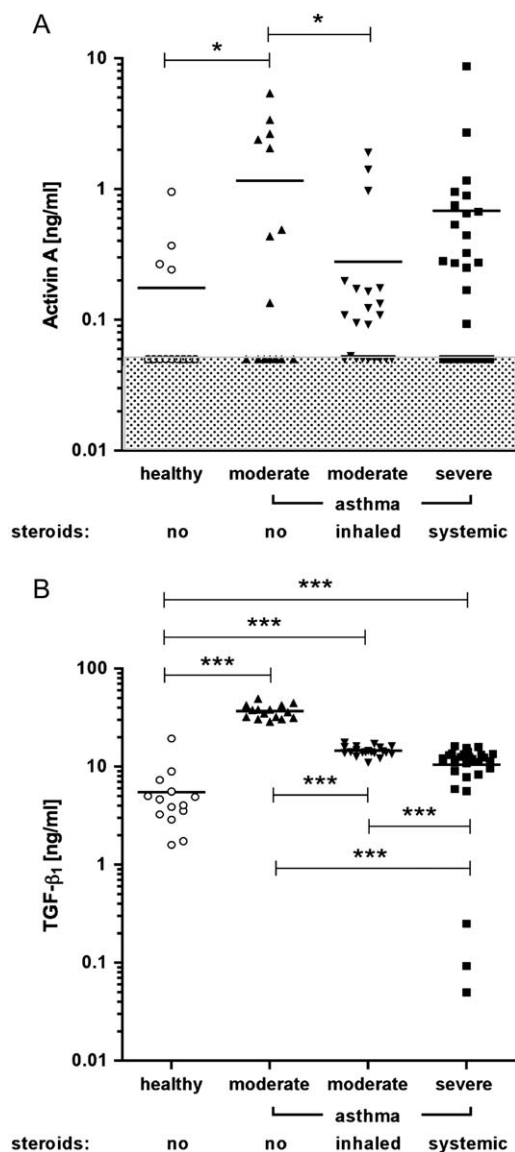


FIG 1. A, Activin A serum levels are increased in patients with moderate asthma ($n = 15$) who were not treated with steroids, whereas patients with moderate asthma with inhaled GC treatment ($n = 22$) and patients with severe asthma ($n = 29$) did not show increased levels. **B,** TGF- β_1 serum levels are increased in both patients with moderate asthma with ($n = 20$) and without ($n = 15$) steroid treatment and in patients with severe asthma ($n = 29$). The dotted area in Fig 1, A, indicates our detection limit of the activin A ELISA. Statistical significance is indicated by asterisks (* $P \leq .05$, ** $P \leq .01$, *** $P \leq .001$).

0.163 ng/mL, $n = 29$). In the same population patients with moderate and severe asthma showed increased serum levels of TGF- β_1 compared with healthy control subjects ($P \leq .001$; Fig 1, B). Furthermore, patients without GCs showed the highest TGF- β_1 serum levels compared with those of patients with moderate asthma with GCs or patients with severe asthma ($P \leq .001$). Patients included in the study with moderate asthma received no drugs or inhaled GCs, as indicated in Fig 1, whereas patients with

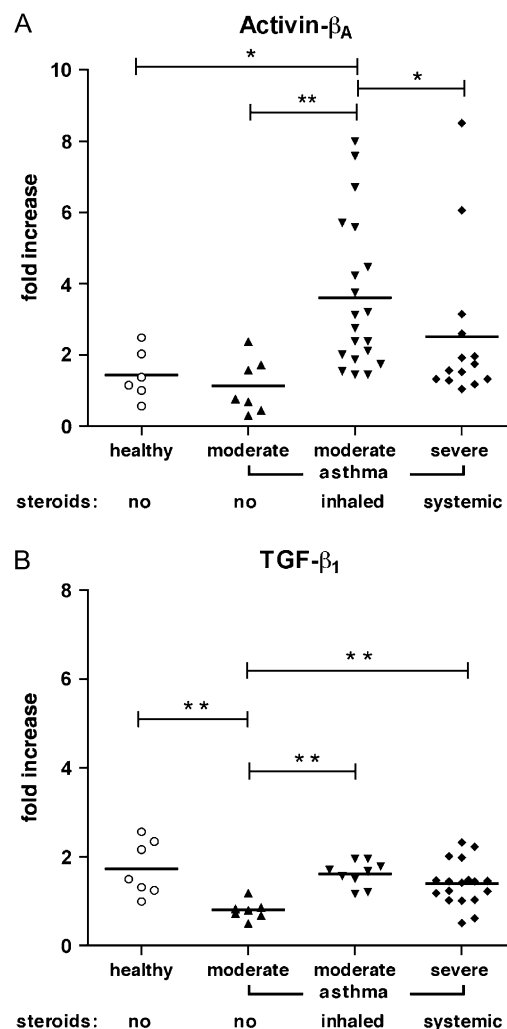


FIG 2. A, Activin β_A mRNA expression, measured by means of real-time PCR, is increased in freshly isolated $CD4^+$ T cells of patients with moderate asthma treated with GCs ($n = 20$) compared with that seen in patients with moderate asthma without steroid treatment ($n = 7$), patients with severe asthma ($n = 14$), or healthy control subjects ($n = 6$). **B,** In contrast, TGF- β_1 mRNA expression is decreased in $CD4^+$ T cells of patients with moderate asthma without GCs compared with that seen in healthy subjects and patients with severe asthma. Data were normalized to one randomly chosen healthy donor and set as 1. Statistical significance is indicated by asterisks (* $P \leq .05$, ** $P \leq .01$).

severe asthma received inhaled and systemic GCs. Of note, serum levels for TGF- β_1 are more than 20-fold higher than those for activin A (mean TGF- β_1 level: healthy subjects, 5.49 ± 1.19 ng/mL; patients with moderate asthma without GCs, 36.78 ± 1.57 ng/mL; patients with moderate asthma with inhaled GCs, 14.51 ± 0.37 ng/mL; patients with severe asthma, 11.72 ± 0.53 ng/mL).

Activin β_A , but not TGF- β_1 , mRNA expression is increased in $CD4^+$ T cells of asthmatic patients

Because $CD4^+$ T cells play a major role in the pathogenesis of asthma, we investigated the expression of

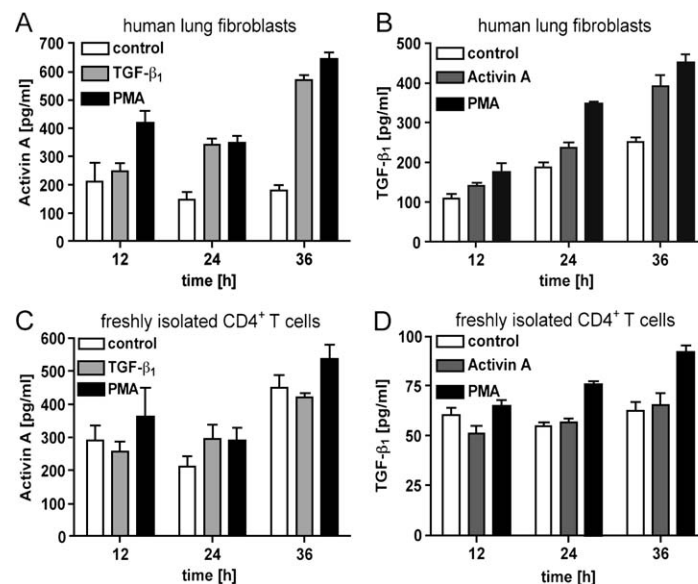


FIG 3. TGF- β_1 (5 ng/mL) induces activin A in a noncancer human lung fibroblast cell line (IMR-90; **A**). In turn, activin A (25 ng/mL) only weakly induces TGF- β_1 in IMR-90 (**B**) cells. In freshly isolated CD4⁺ T cells from healthy donors, TGF- β_1 could not induce activin A (**C**), and activin A could not induce TGF- β_1 (**D**). PMA (100 ng/mL) was used as a positive control. Data are representative of 4 independent experiments. Error bars indicate the SD of each experiment.

TGF- β_1 and activin βA in CD4⁺ T cells of asthmatic patients. We have previously shown, by means of cDNA array analysis, that CD4⁺ T cells from asthmatic patients and activated *ex vivo* express more activin βA mRNA than those from healthy control subjects.³³ To confirm these results and to determine whether TGF- β_1 levels were concomitantly increased in CD4⁺ T cells from patients with asthma, we used quantitative real-time PCR to determine activin βA and TGF- β_1 mRNA expression in CD4⁺ T cells in asthmatic patients and healthy non-allergic control subjects. Activin βA mRNA expression in freshly isolated CD4⁺ T cells was significantly increased (3.6-fold increase [range, 2.6- to 4.6-fold increase], $P \leq .05$; Fig 2, **A**) in patients with moderate asthma taking inhaled GGs ($n = 20$) compared with that in healthy control subjects ($n = 6$), patients with moderate asthma without GCs ($n = 7$), or patients with severe asthma ($n = 14$). In contrast, we found no significant difference between the TGF- β_1 mRNA expressed in CD4⁺ T cells between the treated groups compared with that seen in healthy control subjects ($P > .05$; Fig 2, **B**). Only patients without drug treatment showed decreased TGF- β_1 mRNA expression. Activin A protein expression was confirmed by means of ELISA and immunohistochemical staining of cytospin preparations of CD4⁺ T cells (Fig 3, **C**, and data not shown). Partially in contrast to the *in vivo* findings, dexamethasone could reduce activin βA mRNA expression in *in vitro* cultures of CD4⁺ T cells (data not shown).

Activin βA is expressed in lungs of mice with allergic asthma

During early allergen-induced and T cell-dependent inflammation *in vivo*, we investigated the expression

of activin A in the lungs of mice sensitized and aerosolized on 2 separate occasions with OVA.³⁰ We stained paraffin-embedded sections of mice after a secondary aerosol OVA challenge compared with healthy control animals and recovered mice with anti-activin A. Activin A was similarly detected in epithelium of naive, recovered, and OVA-rechallenged mice (Fig 4, **A** and **B**). Additionally, we observed that activin A was concentrated in leukocytes infiltrating OVA-rechallenged lungs. To determine changes in activin βA mRNA expression after early aerosol OVA rechallenge, we harvested lungs 2 and 12 hours after two or four 1-hour OVA nebulizations (see the Methods section). Real-time PCR analysis of total lung RNA revealed a 4-fold increase in activin A expression 12 hours after two 1-hour and four 1-hour OVA challenges. In contrast, expression of activin βA mRNA was not increased 2 hours after two or four 1-hour aerosol OVA challenges (Fig 4, **C**). In contrast to activin βA , no significant increase in TGF- β levels after the OVA challenges was observed (Fig 4, **D**).

Induction of activin A by TGF- β_1 in human lung fibroblasts

Phorbol myristate acetate (PMA) is known to be a strong inducer of activin A expression.²⁶ TGF- β_1 and PMA are able to induce activin A in cell cultures of human lung fibroblasts (IMR-90) in a time- and dose-dependent manner (Fig 3, **A**). Induction of activin A protein by TGF- β_1 was 3-fold higher than the basal level of activin A produced by fibroblasts. Activin A could induce TGF- β_1 in the same cell-culture system, although with a slower and weaker progress and maximal levels after 36 hours (Fig 3, **B**). In contrast, TGF- β_1 could not induce activin

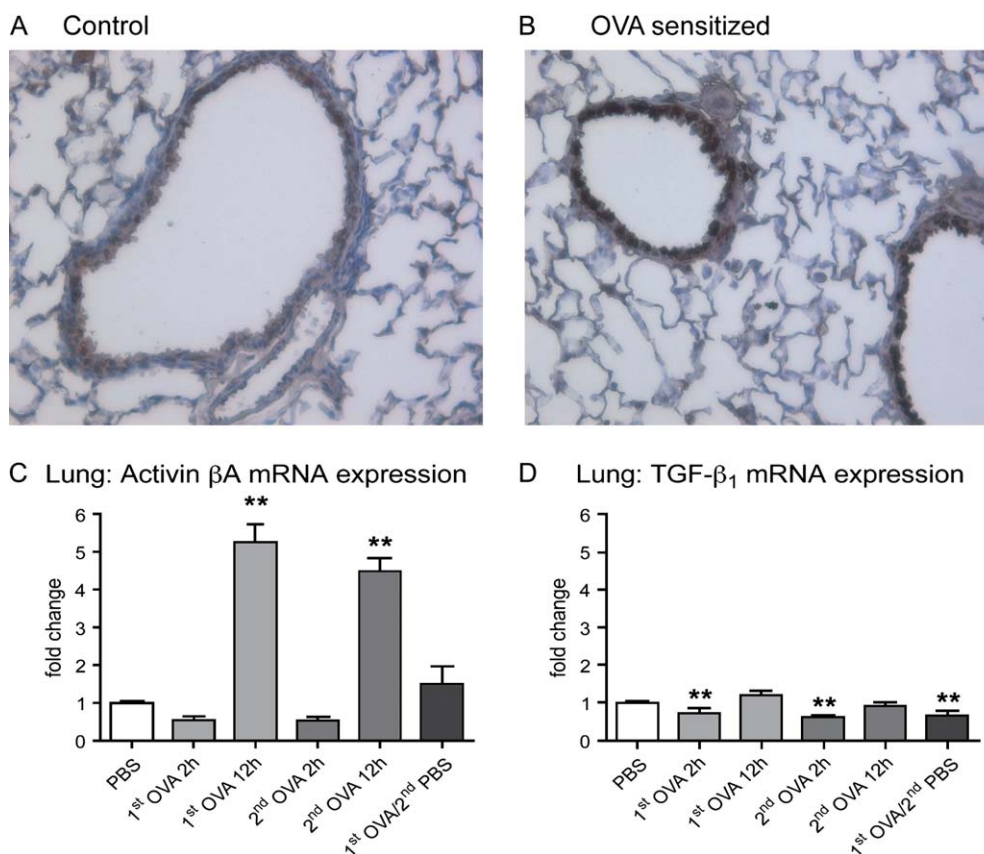


FIG 4. Immunohistologic staining of activin β A. Lung sections of OVA-sensitized mice treated with aerosolized PBS (**A**) or OVA (**B**) after 90 days are shown. Quantitative analysis of gene expression by means of real-time PCR of activin β A and TGF- β ₁ mRNA in total lungs of OVA-sensitized and nonsensitized mice, as indicated, are shown in **C** and **D**. The figures represent 3 independent experiments. Statistical significance is indicated by asterisks (**P ≤ .01). Error bars indicate the SD of each experiment.

A and activin A could not induce TGF- β ₁ in freshly isolated CD4⁺ T cells from healthy donors (Fig 3, C and D). Of note, the same concentrations of PMA were not sufficient to induce activin A or TGF- β ₁ in CD4⁺ T cells, as they were in human lung fibroblasts. To analyze as closely as possible the expression of activin β A in the *in vivo* environment, precisely cut slices taken from different areas of a human lung were cultivated in triplicate, with and without the addition of T_H1 and T_H2 cell supernatants (see this article's Fig E2, A and B, in the Online Repository at www.jacionline.org). T_H1 and T_H2 cells were both generated from naive CD4⁺ T cells, and IL-4, IL-13 and IFN- γ were quantified by means of ELISA (data not shown). Interestingly, T_H2 supernatants were able to induce the expression of activin β A in human lung tissue (see this article's Fig E2 in the Online Repository at www.jacionline.org). Similarly, primary human lung fibroblasts, cultured in the presence of T_H2 supernatants or IL-13 (see this article's Fig E2, C, and Fig E2, D, respectively, in the Online Repository at www.jacionline.org) also showed a remarkable induction of activin β A (see this article's Fig E2, D, in the Online Repository at www.jacionline.org). In contrast, neither T_H1 supernatants

nor IFN- γ increased activin A secretion. Neither T_H1 nor T_H2 supernatants affected the viability of the fibroblasts (data not shown).

Activin A and TGF- β ₁ enhance fibroblast growth and inhibit T-cell proliferation

Although TGF- β can promote the growth of fibroblasts and the expression of extracellular matrix,⁸ its key regulatory function in immunity is to control proliferation of T cells.⁴ The ability of TGF- β to enhance the proliferation of certain cell lines might depend on the generation of some growth factors.⁸ We observed that activin A and TGF- β enhanced human lung fibroblast proliferation (Fig 5, A and B) and inhibited CD4⁺ T-cell proliferation (Fig 5, C and D). Interestingly, TGF- β was more effective at the same concentrations. These differences might be attributed to differences in the biologic activities of the reagents, to differences in the densities of the ligands' receptors, or to Smad-independent pathways of TGF- β . To verify the biologic activity of activin A and TGF- β , we introduced a Smad 2/3-responsive reporter gene into IMR-90. Although both activin A and TGF- β induced the Smad 2/3 pathway, activin A required concentrations

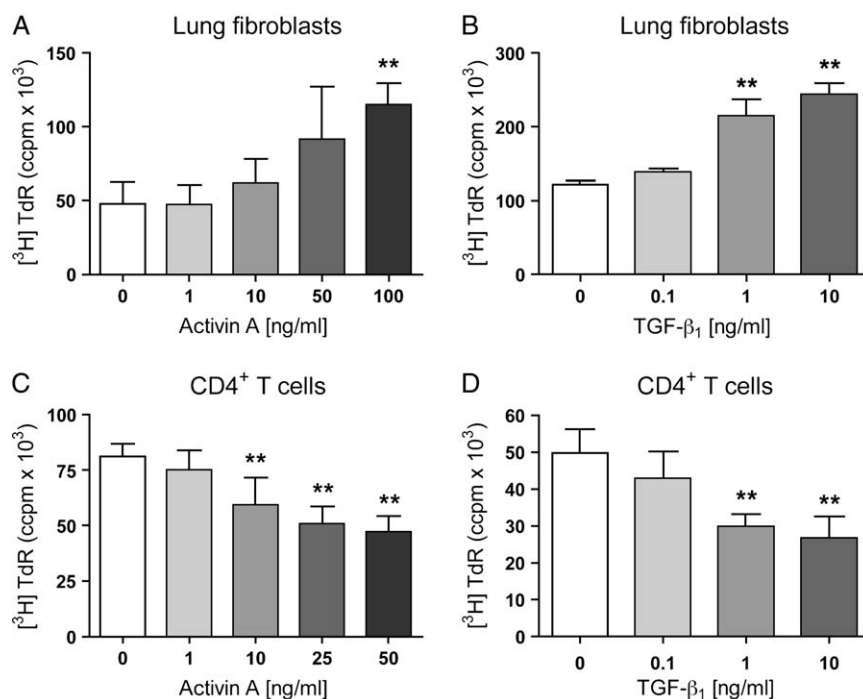


FIG 5. Increased proliferation of the human lung fibroblast cell line IMR-90 in response to activin A (**A**) or TGF-β₁ (**B**). Decreased inhibition of anti-CD3 (5 μg/mL) induced proliferation of CD4⁺ T cells from a healthy donor in response to activin A (**C**) or TGF-β₁ (**D**). The results represent 3 independent experiments. Proliferation was measured by means of tritiated thymidine incorporation for 12 hours. Error bars indicate the SD of each experiment. Statistical significance is indicated by asterisks (***P* ≤ .01).

at least 10 times higher than those needed by TGF-β to reach the same level of Smad activation (see this article's Fig E3 in the Online Repository at www.jacionline.org). Furthermore, although activin A is known to induce apoptosis in several cell types, it could not induce apoptosis under serum-free conditions in CD4⁺ T cells, lung fibroblasts, or airway smooth muscle cells (data not shown).

DISCUSSION

The present study shows that activin A participates in the pathogenesis of asthma. This is documented by increased activin A serum levels in patients with untreated moderate asthma, whereas patients with GC-treated moderate or severe asthma did not show significant differences than healthy control subjects. In contrast, TGF-β₁ serum levels were increased in patients with moderate and severe asthma compared with levels seen in healthy nonallergic control subjects independent of GC treatment. Severity-associated levels of activin A were also reported in recent studies of acute and chronic heart diseases and of acute liver failure.³⁴⁻³⁶ These data show remarkable differences between activin A and TGF-β₁ that might be relevant for the pathogenesis of asthma.

In asthma chronic exposure to allergen is believed to drive the pathogenesis of the lung inflammation. In particular, activated CD4⁺ T cells and increased T_H2 responses³⁷ represent a cardinal feature of asthma, but the

link to TGF-β₁-dependent remodeling of the lung is not yet known. We therefore investigated activin A, which has been suggested to be involved in remodeling,^{27,38} and its expression in CD4⁺ T cells. In contrast to severity-associated activin A serum levels, only patients with moderate asthma showed increased activin A mRNA expression in CD4⁺ T cells, whereas healthy control subjects and patients with severe asthma did not. This lack of activin A mRNA expression increase in CD4⁺ T cells seen in patients with severe asthma might be due to the systemic steroid treatment because GCs could decrease activin A mRNA expression in *in vitro* cultures of CD4⁺ T cells. Furthermore, TGF-β₁ mRNA expression was the same in healthy control subjects and patients with moderate or severe asthma. Furthermore, the results suggest that activin A expression in CD4⁺ T cells is allergen inducible and GC sensitive, which we confirmed *in vitro* by adding allergens to freshly isolated PBMCs (data not shown). In a murine model of allergic asthma, activin A-expressing T cells were observed in peribronchial infiltrates after allergen provocation (data not shown).

Moreover, the study demonstrates that activin A mRNA, but not TGF-β₁ mRNA, is locally inducible by allergen exposure, which we also could confirm by means of *in vitro* studies with human PBMCs. The inhalant allergen-mediated induction of activin A was reinducible, whereas TGF-β₁ remained at the same levels. In contrast to TGF-β₁, activin A was also secreted by epithelial cells of control mice and healthy human subjects, whereas

epithelial expression of TGF- β could not be observed in bronchial biopsy specimens of healthy human subjects.³⁹ The missing increase in local TGF- β_1 expression might be due to a different time point because it could be shown that repeated OVA challenge increased TGF- β_1 expression⁴⁰ and peribronchial TGF- β_1 -expressing eosinophils.⁴¹ Local homeostatic activin levels can be increased by T_H2, but not T_H1, T-cell supernatants, confirming previous studies showing that T-cell cytokines can induce activin A in monocytes and stromal fibroblasts.²⁰ This suggests an important role of activin A in T_H2-dependent allergic reactions. Interestingly, T_H2 cytokines failed to induce activin A expression in CD4⁺ T cells (data not shown), suggesting that the expression of activin A is subjected to different gene regulation in lymphoid cells.

We show that activin A can induce TGF- β_1 in fibroblasts and that TGF- β_1 induces activin A in an even stronger manner. Therefore activin A provides a link between allergen-specific T-cell responses and TGF- β_1 -mediated remodeling processes. The current data confirm previous studies showing that activin A displays similar dichotomous functions like TGF- β_1 , such as increased fibroblast proliferation^{8,27,38} and suppressed lymphocyte proliferation.⁴² However, higher concentrations of activin A are needed to obtain similar effects compared with TGF- β_1 . The higher potency of TGF- β_1 is reflected by more abundant mobilization of the Smad 2/3 pathway. This suggests an interdependent network of both cytokines, in which TGF- β_1 is the key profibrotic factor in the chronic phase and activin A is the regulator cytokine in the early immune response. Current investigations are initiated that focus on this hypothesis through selective elimination of activin A or TGF- β_1 .

Taken together, we demonstrate that activin A is linked to TGF- β_1 and thus to remodeling, which is a key feature of asthma. In the network of activin A and TGF- β_1 regulation, activin A appears to play the role of immediate response regulator and might act as a modulator of the balance between tissue integrity and remodeling processes toward remodeling.

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