Activin-A Overexpression in the Murine Lung Causes Pathology That Simulates Acute Respiratory Distress Syndrome

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Rationale: Activin-A is up-regulated in various respiratory disorders. However, its precise role in pulmonary pathophysiology has not been adequately substantiated in vivo.

Objectives: To investigate in vivo the consequences of dysregulated Activin-A expression in the lung and identify key Activin-A–induced processes that contribute to respiratory pathology.

Methods: Activin-A was ectopically expressed in murine lung, and functional, structural, and molecular alterations were extensively analyzed. The validity of Activin-A as a therapeutic target was demonstrated in animals overexpressing Activin-A or treated with intratracheal instillation of LPS. Relevancy to human pathology was substantiated by demonstrating high Activin-A levels in bronchoalveolar lavage (BAL) samples from patients with acute respiratory distress syndrome (ARDS).

Measurements and Main Results: Overexpression of Activin-A in mouse airways caused pulmonary pathology reminiscent of acute lung injury (ALI)/ARDS. Activin-A triggered a lasting inflammatory response characterized by acute alveolar cell death and hyaline membrane formation, sustained up-regulation of high-mobility group box 1, development of systemic hypercoagulant state, reduction of surfactant proteins SpC, SpB, and SpA, decline of lung compliance, transient fibrosis, and eventually emphysema. Therapeutic neutralization of Activin-A attenuated the ALI/ARDS-like pathology induced either by ectopic expression of Activin-A or by intratracheal instillation of LPS. In line with the similarity of the Activin-A–induced phenotype to human ARDS, selective up-regulation of Activin-A was found in BAL of patients with ARDS.

Conclusions: Our studies demonstrate for the first time in vivo the pathogenic consequences of deregulated Activin-A expression in the lung, document novel aspects of Activin-A biology that provide mechanistic explanation for the observed phenotype, link Activin-A to ALI/ARDS pathophysiology, and provide the rationale for therapeutic targeting of Activin-A in these disorders.

Keywords: Activin-A; ALI; ARDS; inflammation; mouse model

Acute lung injury (ALI) and its most severe form, acute respiratory distress syndrome (ARDS), are fulminating respiratory conditions in which injury of lung epithelium and endothelium, accompanied by uncontrolled inflammatory activity, often result in marked functional and structural alterations of the lung (1–3). ARDS is among the leading causes of death in ICUs, constituting a major burden to health care systems worldwide (3–6). Identification of rate-limiting components of the chaotic dysregulation of immune reactivity in this disease is essential for development of effective treatment strategies (3, 6).
Activin-A, a member of the transforming growth factor (TGF)-β superfamily, originally identified as a regulator of follicle-stimulating hormone secretion by the anterior pituitary gland (7), has attracted renewed attention due to its implication in several facets of immunity and tissue repair (8–11). Functionally, Activin-A is characterized by a remarkable complexity and numerous studies have suggested apparently conflicting activities for this molecule. A proinflammatory role has been supported by in vitro studies demonstrating that Activin-A induces IL-6, tumor necrosis factor (TNF)-α, IL-1β, and inducible nitric oxide synthase (iNOS) expression in macrophages (12). Conversely, an antiinflammatory role has been supported by studies demonstrating Activin-A–mediated suppression of IL-6–mediated signaling (13), secretion of IL-1 receptor antagonist (14) and NO synthesis by LPS-activated macrophages (12, 15), dendritic-cell maturation (16), development of Foxp3⁺/IL-10–producing regulatory CD4⁺ T cells (17), and, in synergy with TGF-β, development of Foxp3⁺ regulatory cells (18). Activin-A has also been implicated in the regulation of tissue-repair and matrix-remodeling related processes (19–25). Not surprisingly, considering such a broad spectrum of biological activities, both harmful and protective roles have been postulated for Activin-A in the context of respiratory pathology (17, 26). However, its precise role in pulmonary pathophysiology has not been adequately substantiated in vivo as yet.

In this study, we demonstrate that overexpression of Activin-A in murine lung leads to respiratory pathology that bears striking similarity to human ALI/ARDS, and importantly, we show that Activin-A is up-regulated in bronchoalveolar lavage (BAL) fluids of patients with ARDS, at levels comparable to those induced in our animal model. Furthermore, we document for the first time the role of Activin-A in biological processes relevant to lung physiology, including regulation of surfactant-protein (Sp) synthesis and development of a procoagulant state. Lastly we show that therapeutic neutralization of Activin-A attenuates pathology caused either by ectopic or endogenous Activin-A expression, strongly suggesting that Activin-A inhibitors may provide therapeutic benefit to patients with ALI/ARDS.

**METHODS**

**Animals**

C57BL/6 female mice, 12–16 weeks of age, were purchased from Charles River Laboratories (Calco, Italy) and maintained in individually ventilated cages in temperature-controlled rooms (23 ± 1°C) under 12-h light/dark cycle and ad libitum access to food and water. All procedures had received approval from Institutional and Regional Ethical Review Boards.

**Intratracheal Instillation of Recombinant Adenovirus**

A total of 8 × 10⁸ infectious particles of adenoviruses expressing human Activin-A and green fluorescent protein (Ad-ActA) or green fluorescent protein only (Ad-GFP), corresponding to ∼1.5 × 10⁸ plaque-forming units (see MATERIÀLS AND METHODS in the online supplement) diluted in 40 μl phosphate-buffered saline were instilled intratracheally into anesthetized mice. For in vivo Activin-A neutralization, 200 μg/injection/animal of either a soluble ActRIIB-Fc fusion-protein or a control human IgG1-Fc fragment (BioXcell, West Lebanon, NH) were injected intraperitoneally as outlined in Figures 1 and 8.

**Human BAL Samples**

BAL fluid was collected from 21 mechanically ventilated patients with ARDS (27) in the ICUs of Evangelismos Hospital, Athens (n = 14), and University Hospital, Alexandroupolis (n = 7). Details on the method for BAL-sample preparation are provided in the online supplement and patient characteristics are provided in Table E2 in the online supplement.

**Statistics**

Data were analyzed using one-way analysis of variance with Bonferroni’s post hoc test analysis. Due to minimal variations in the values of the untreated animals in Figures 4B and 5A, the mean values of each

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**Figure 1.** Adenovirus-mediated Activin-A overexpression in the lung. (A) Experimental protocol for viral instillation and Activin-A neutralization using ActRIIB-Fc. Viruses expressing human Activin-A and green fluorescent protein (Ad-ActA) or green fluorescent protein only (Ad-GFP) were instilled in the trachea of C57BL/6 female mice, which were analyzed up to 120 days postinstillation. For prophylactic or therapeutic neutralization of Activin-A, 200 μg of recombinant ActRIIB-Fc were injected intraperitoneally on the indicated days and the animals were analyzed on Day 15. (B) Activin-A protein concentration in bronchoalveolar lavage fluids and follistatin mRNA levels in total lung extracts from Ad-ActA–treated (n = 5 per time point, open circles), Ad-GFP–treated (n = 3 per time point, closed circles), and untreated control animals (n = 3 per time point, open triangles). Virus-treated groups were compared with the untreated “pool” control group using one-way analysis of variance with Bonferroni’s post hoc test analysis. *P < 0.05, **P < 0.01, and ***P < 0.001. BAL = bronchoalveolar lavage; BALF = bronchoalveolar lavage fluid; GAPDH = glyceraldehyde phosphate dehydrogenase; mRNA = messenger RNA.
Activin-A overexpression in Airways Causes Severe and Lasting Respiratory Pathology

Activin-A was expressed in the airways of C57BL/6 mice by adenovirus-mediated gene transfer and animals were studied for up to 4 months (Figure 1A). Analysis of BAL fluids demonstrated homeostatic levels of Activin-A throughout the experiment in untreated and Ad-GFP–treated animals (~150 and ~300 pg/ml, respectively). In the Ad-ActA group, increased expression of Activin-A was evident from Day 2 (~5 ng/ml), peaked on Day 7 (~28 ng/ml), and despite a gradual decline, remained high up to Day 56 (Figure 1B). Substantial increase in mRNA levels of follistatin, the natural inhibitor of Activin-A, was evident on Day 7, reached maximum levels around Day 24, and persisted till Day 34 (Figure 1B).

Ad-GFP–treated animals exhibited typical adenovector kinetics, with GFP-mRNA reaching maximum expression around Day 7 and disappearing around Day 15. The prolonged expression of virus-encoded Activin-A could indicate attenuation of the adaptive immune responses that normally clear adenovirus and may reflect the reported capacity of Activin-A to influence directly or indirectly development of T-regulatory cells (17, 18).

The physiological sequelae of Activin-A overexpression were evaluated by measuring static (Cst) and dynamic (Cdyn) compliance and central airway (Rn) and total lung (R) resistance. In Ad-ActA–treated animals, Cst decreased gradually to ~40% below baseline by Day 24, remained low until Day 56, and was partially normalized at 4 months. Rn increased in Ad-ActA–treated animals; however, the change was less dramatic than in Cst (Figure 2A). Changes in Cdyn and R followed those of Cst (data not shown).

Evaluation of hematoxylin and eosin–stained lung sections demonstrated sustained alveolar remodeling in Ad-ActA–treated animals (Figure 2B). From Day 2 and reaching maximum around Day 24, alveolar septa were gradually thickened without, however, distorting grossly lung architecture. Between Days 24 and 34, however, a heterogeneous histopathology emerged with lung fields exhibiting thickened alveolar walls with honeycombing-like morphology often adjacent to regions of thinning and fibrillation of the alveolar walls with a typical hyperinflation and emphysematous-type morphology. Disorganization of elastic fibers and connective tissue elements and a higher incidence of hyaline-membrane formation was associated with areas of alveolar edema (Figure E1A). From Day 35 on, the number of cells infiltrating alveolar septa declined, whereas regions with alveolar wall disorganization/fibrillation and hyperinflation became the dominant histological feature that persisted up to 4 months.

Analysis of parenchymal sections of Ad-ActA–treated animals demonstrated transient increase in αSMA immunostaining (maximum at ~Day 24) and collagen deposition (maximum at ~Day 34) and gradual increase in mean alveolar surface, which persisted until the end of the experiment (Figure E2). Collagen accumulation was associated with increased mRNA levels for collagen-I and -III, tissue inhibitor of metalloproteinase-1, and connective tissue growth factor, whereas resolution of collagen deposition and development of emphysematous lesions correlated with decline of tissue inhibitor of metalloproteinase-1 and collagenase increase in MMP2 and MMP9 mRNA levels (Figure E2C). The transient fibrotic response did not correlate with
expression is manifested as intense nuclear and diffuse cytoplasmic staining in inflammatory cells, extracellular staining in the parenchyma, and, notably, in the lumen of vascular capillaries. Interstitial and intracapillary staining is shown with arrows and arrowheads, respectively.

any obvious increase in either mRNA or protein TGF-β level (Figures E2C and E3). Only minor inflammatory changes consisting essentially of inflammatory cell occupation of the adventitia of pulmonary arteries and arterioles were evident in Ad-GFP–treated animals (Figure E4A).

**Activin-A-induced Lung Injury Is Associated with Acute Alveolar Cell Death, Sustained Up-Regulation of High-mobility Group Box 1, and Inflammation**

To delineate whether the alveolar wall damage involved apoptotic cell death, tissue sections were analyzed by TUNEL assay. A dramatic wave of TUNEL signal that peaked 48 hours after Ad-ActA instillation was observed, while levels of Activin-A in the BAL fluids were approximately 5 ng/ml (Figure 3). Thereafter, despite further increase of Activin-A to ~28 ng/ml, TUNEL+ staining drastically declined (Figure 3C). Interestingly, few TUNEL+ cells exhibited typical apoptotic cell morphology. The majority of the staining involved fragmented cell debris either attached to basement membranes of alveolar septa or present in clumps in airspaces (Figures 3A and 3B). This staining pattern could stem from a rapid transition of cells undergoing apoptosis into secondary necrosis and cellular rupture.

Costaining for CD45, T1α, SpC, and TUNEL demonstrated that TUNEL+ material in airspaces was admixed mainly with T1α-positive fragments (Figure E5), suggesting that it was derived from type I pneumocyte fragmentation. Occasionally, some TUNEL+ type II pneumocytes were also detected in affected areas. No TUNEL+ material was detected in lungs of untreated or Ad-GFP–treated animals (Figure 3C).

Rupture of damaged cells could lead to release of damage-associated molecular patterns (DAMPs), which are well recognized in experimental animal models and human diseases as key proinflammatory “drivers” (28). Therefore, we investigated if overexpression of Activin-A was associated with alterations in the level or pattern of expression of high-mobility group box (HMGB) 1, the prototypical DAMP molecule. Indeed, Ad-ActA–treated animals demonstrated substantial increase of HMGB1 immunostaining that was apparent already on Day 2, reached maximum levels around Day 15, and persisted until around Day 56 (Figure 3D).

Activin-A overexpression triggered a dynamically evolving inflammatory response that consisted of neutrophils and macrophages (Days 0–4), macrophages, neutrophils, and lymphocytes (Days 5–15), and macrophages and lymphocytes (Days 15–56) (Figure E6A). Inflammation was accompanied by a dynamically evolving “cytokine-storm”–like response with monocyte chemotactic protein-1/C-C motif ligand-2 and IL-6 mRNA (maximum levels at ~Days 2–7), IL-1β, IL-4, KC/IL-8, IFNγ, TNFα, and IL-17 (maximal levels at ~Day 7), and IL-5 and IL-10 (maximum levels at ~Day 15). After Day 15, a rapid decline in cytokine/chemokine mRNA synthesis was observed, with the exception of a second wave of KC/IL-8 expression and a further increase in the levels of IL-17α mRNA (Figure E6B). Selected cytokines, namely, monocyte chemotactic protein-1/C-C motif ligand-2, IL-6, IL-5, and TNFα were analyzed at the protein level, confirming their up-regulation with kinetics similar to the mRNA (Figure E6E).

**Activin-A Alters Composition and Function of Lung Epithelium**

Although Clara cells dominated distal airways in untreated and Ad-GFP–treated animals, the majority of airway epithelial cells in Ad-ActA–treated animals between Days 15 and 35 were ciliated (Figure E7A). In the alveolar compartment, numbers...
of type I pneumonocytes gradually declined (Figure E7B) and clusters of detached T1α+ pneumonocytes were found in airspaces. These T1α+ cells were TUNEL negative, and thus, apoptosis as a mechanism of their reduction was deemed unlikely.

Type II pneumonocytes were drastically affected by Activin-A. SpC immunostaining declined rapidly, reached lowest levels around Day 7, and remained low until Day 15 (Figure 4A). Thereafter, SpC+ cells reappeared in clusters, usually in regions neighboring those severely affected. Even on Day 56, SpC expression was markedly heterogeneous, with areas of the lung containing high numbers of SpC+ cells, whereas others areas were completely devoid of signal.

Figure 4. Down-regulation of the surfactant protein (Sp) levels in Ad-ActA–treated animals. (A) Confocal images from lung tissues immunostained for SpC at the indicated time points. The regional heterogeneity of SpC expression pattern at the later stages of the Activin-A–induced response is illustrated by the inclusion of two neighboring images (Days 34 and 56) with representative SpC–devoid and SpC-rich regions (the border between SpC–devoid and SpC-rich regions is shown with the dashed line). SpC immunostaining was pseudocolored white. Cells stained green are virus-infected GFP–expressing cells. (B) Quantitative polymerase chain reaction (PCR) analysis of surfactant protein messenger RNA (mRNA) expression in the Ad-ActA, Ad-GFP, and untreated groups. The Ad-ActA (n = 5 per time point, open circles) and Ad-GFP (n = 3 per time point, closed circles) virus–treated groups were compared with the untreated "pool" control group using one-way analysis of variance with Bonferroni’s post hoc test analysis. *P < 0.05, **P < 0.01, and ***P < 0.001. (C) Quantitative PCR analysis of the mRNA levels of SpC and SpB in the mouse lung epithelial cell line MEL12 after 24 h culture with recombinant Activin-A (10 ng/ml) and/or follistatin (30 ng/ml). Values represent mean ± SEM of four independent experiments. The groups were compared using one-way analysis of variance with Bonferroni’s post hoc test analysis. *P < 0.05, **P < 0.01, and ***P < 0.001. ActA = Activin-A; Fst = follistatin; GAPDH = glyceraldehyde phosphate dehydrogenase; UT = untreated.
mRNA levels for SpC, SpB, and SpA were markedly downregulated in Ad-ActA–treated animals (Figure 4B). Trough levels were around Days 7–15, reduced approximately 70–80% compared with Ad-GFP and untreated controls. The effect of Activin-A was selective, as SpD levels were not reduced at any time point but rather demonstrated statistically significant increase around Days 24–34. Down-regulation of SpC and SpB mRNA levels could stem from direct action of Activin-A on epithelial cells as the signal could be recapitulated \textit{in vitro} by treating the epithelial cell lines MLE12 (Figure 4C) and MLE15 (data not shown) with 10 ng/ml recombinant Activin-A.

\section*{Activin-A Overexpression Leads to Systemic Procoagulant State}

Inflammatory processes in the lung can promote a procoagulant state (29). Therefore, we investigated the effect of Activin-A overexpression on hemostatic balance using a “tail-bleeding-time” assay (Figure 5A). Bleeding time in Ad-ActA–treated animals 7 days after viral instillation was reduced approximately 75%. Bleeding time gradually improved, reaching normal levels at 4 months. Kinetics of tail-bleeding-time reduction correlated with an increase in mRNA levels for tissue factor (TF), a key activator of the extrinsic coagulation pathway (30), suggesting that up-regulation of TF could be one of the mechanisms by which Activin-A interferes with the coagulation cascade. Up-regulation of TF RNA levels could stem from direct action of Activin-A on epithelial cells, because it could be recapitulated \textit{in vitro} by treating the epithelial cell lines MLE12 (Figure 5B) and MLE15 (data not shown) with 10 ng/ml recombinant Activin-A.

\section*{Increased Levels of Activin-A in BAL Fluid of Patients with ARDS}

When considered in isolation the changes observed in Ad-ActA–treated animals resemble those seen in different human respiratory disorders. However, when considered in aggregate, they bear marked similarities to those described in ARDS (1). Therefore, we analyzed Activin-A and follistatin expression in BALs from patients with ARDS. Levels of Activin-A in the group of patients with ARDS were significantly higher than the control group (Figure 6A). Considering the significant dilution of BAL fluids during collection (~25–100 fold, estimated from protein concentrations in BAL and serum), BAL Activin-A levels of 2–3 ng/ml suggest that local concentration of this molecule in affected areas could reach and even exceed the levels of ectopically expressed Activin-A in our experimental animals.

Follistatin was also elevated in ARDS, although pairwise comparison of Activin-A and follistatin levels in the same patient revealed that in all but one, the concentration of Activin-A exceeded that of follistatin (Figure 6B). Since BAL cells from some patients with ARDS were also available as frozen samples, mRNA levels of Activin-A, Activin-B, and TGF-\(\beta_1\) were analyzed (Figure 6C). In comparison to peripheral blood mononuclear cells from healthy donors, BAL cells from patients with ARDS contained approximately 40 times more Activin-A mRNA. This increase appears to be selective, because the same samples contained marginally elevated Activin-B and only five-fold to sixfold higher TGF-\(\beta_1\) mRNA.

\section*{Therapeutic Neutralization of Activin-A Protects Animals from Activin-A–induced Pathology}

Because in several experimental models Activin-A was neutralized by “prophylactic” delivery of follistatin, the question whether Activin-A neutralization is beneficial once pathology has developed was still open. To validate Activin-A as a therapeutic target, we used a fusion protein composed of the extracellular portion of the Activin type II receptor ActRIIB fused to the Fc portion of human IgG1 (see MATERIALS AND METHODS in the online supplement). Although all Ad-ActA–treated groups contained similar levels of Activin-A mRNA in their lungs (Figure 7C), those treated either prophylactically or therapeutically exhibited dramatic normalization of lung compliance, tail-bleeding time, histology, and mRNA levels of SpC, IL-6, collagen-I and -III, TF, and Arginase-1 (Figure 7).

To further validate the role of Activin-A in ALI/ARDS pathophysiology, we utilized an established murine model in which only endogenous Activin-A production is involved, the acute lung injury induced by instillation of LPS in the lung. Animals were treated with 2.5 mg/kg LPS intratracheally and analyzed 5 days later. LPS-treated animals had increased levels of Activin-A in BAL (~3–4 ng/ml BAL) and exhibited dramatic, predominantly neutrophilic inflammation, substantial reduction in lung compliance, reduction in SpC immunostaining, systemic procoagulant state, and severe tissue injury as illustrated by the abundance of TUNEL staining (Figure 8). Prophylactic and therapeutic neutralization of endogenous Activin-A with ActRIIB-Fc protein reduced Activin-A protein levels, improved lung compliance and tail-bleeding time (Figure 8A), reduced dramatically TUNEL staining, up-regulated SpC immunostaining (Figure 8B), and reduced...
neutrophilic inflammation and levels of IL-6 protein in the BAL (Figure E9), strongly supporting the notion that this cytokine could be a key player in the pathophysiology of ALI/ARDS.

**DISCUSSION**

To validate directly the consequences of deregulated Activin-A expression in the respiratory system in vivo, we expressed Activin-A in mouse airways using adenoviral-mediated gene transfer and monitored functional and structural alterations in the respiratory system. Our studies demonstrate that sustained up-regulation of Activin-A, at levels comparable to those measured in patients with ARDS, induces severe and continuously evolving respiratory pathology. The process begins with a wave of acute alveolar-cell death and is followed by waves of inflammatory cells and soluble mediators. High levels of HMGB1, the archetypal DAMP protein, accompany pathology through its entire duration. A systemic procoagulant state develops that correlates with up-regulation of TF mRNA levels. Additionally, Activin-A causes reduction of type I pneumocytes and levels of SpC, SpB, and SpA. The later phase of Activin-A–induced response is characterized by decline in production of proinflammatory and increase in expression of antiinflammatory cytokines, a shift of macrophages toward an “alternative-activated” phenotype, and induction of a transient fibrotic response. Clearance of collagen deposits that correlates with a shift in protease/antiprotease balance in favor of the former and eventually development of emphysematous lesions is the final morphological phenotype. The severe tissue damage, the chronic up-regulation of HMGB1 expression, the mobilization of neutrophils and macrophages, the profile and kinetics of the induced cytokines, the reduction of SpC, SpB, and SpA expression, the alveolar edema and the deposition of hyaline membranes on alveolar walls, the transient fibrotic response, and the eventual accumulation of

**Figure 7.** Prophylactic and therapeutic neutralization of Activin-A protects experimental animals from the Activin-A–induced pathology. ActRIIB-Fc recombinant protein was injected intraperitoneally (200 μg/injection) in Ad-ActA–treated animals following a prophylactic (AdActA/ActRFc-P) or therapeutic (AdActA/ActRFc-T) protocol as outlined in Figure 1A. As control, equal amounts of recombinant Fc fragment of human IgG1 were injected in Ad-ACTA–treated animals (AdActA/Fc-P and AdActA/Fc-T, respectively). (A) Lung compliance and tail-bleeding time were analyzed on Day 15 as described in METHODS. (B) Representative hematoxylin and eosin–stained sections from animals that received prophylactic or therapeutic administration of ActRIIB-Fc or control human IgG1-Fc proteins. (C) Messenger RNA levels of Activin-A, surfactant protein C, IL-6, collagen-I, tissue factor, and Arg1. Values are expressed as mean ± SEM of eight animals per group compared using one-way analysis of variance with Bonferroni’s post hoc test analysis. *P < 0.05, **P < 0.01, and ***P < 0.001. Arg1 = Arginase-1; Col I = collagen-I; GAPDH = glyceraldehyde phosphate dehydrogenase; SpC = surfactant protein C; TF = tissue factor.
of emphysematous lesions that compose the Activin-A–induced pathology are also recognized in the pathophysiology of human ALI/ARDS (31). This resemblance, combined with detection of substantially increased levels of Activin-A in BAL fluids from patients with ARDS (Figure 6), suggests that Activin-A could play a key role in the pathophysiology of this disorder. Our findings complement earlier studies that have postulated a role for Activin-A in the pathophysiology of sepsis (32). Although ARDS and sepsis are distinct disease entities, there is an intimate interrelation between them (1, 31, 33, 34). A wide variety of precipitating causes for ARDS are recognized; however, severe sepsis is the leading cause of ARDS (1, 27). This “cause–effect” relationship could stem from the mobilization of similar, potentially harmful effector mechanisms during their development, with Activin-A being one of them.

Our studies unveil novel Activin-A–mediated functions that could mechanistically explain its involvement in ARDS pathophysiology, namely, the infliction of severe tissue injury through alveolar epithelial and endothelial cell death, the activation of the coagulation system, the sustained induction of HMGB1 expression, and the reduction of surfactant-protein synthesis. The novel activities of Activin-A described herein are likely to contribute to development of respiratory pathology in several ways. For example, transient wave of cell death and sustained up-regulation of HMGB1 expression are potentially pathogenic. Passive release of HMGB1 by ruptured apoptotic cells and active release of additional HMGB1 from inflammatory cells combined with high levels of produced proinflammatory cytokines could create a highly proinflammatory environment during the early phases of Activin-A–induced response (35). High HMGB1 levels have been detected in patients and experimental models of sepsis, and more importantly, HMGB1 neutralization ameliorated pathology in mouse sepsis models (15). Induction of TF expression and development of a systemic procoagulant state

\[ \text{Figure 8.} \] Prophylactic and therapeutic neutralization of Activin-A attenuates LPS-induced acute lung injury. ActRIIB-Fc recombinant protein was injected intraperitoneally (200 µg/injection) in animals treated by intratracheal instillation of LPS (2.5 mg/kg) following a prophylactic (LPS/ActRFc-P) or therapeutic (LPS/ActRFc-T) protocol as outlined in A. As control, equal amounts of recombinant Fc fragment of human IgG1 were injected in Ad-ActA–treated animals (LPS/Fc-P and LPS/Fc-T, respectively). Activin-A protein levels, lung compliance, and tail-bleeding time were analyzed on Day 4 as described in METHODS. Values are expressed as mean ± SEM of the indicated number of animals derived from two independent experiments and compared using one-way analysis of variance with Bonferroni’s post hoc test analysis. *P < 0.05, **P < 0.01, and ***P < 0.001. (B) Representative H&E-, TUNEL-, and surfactant protein (Sp) C–stained tissue sections from LPS-treated animals treated with ActRIIB-Fc or control human IgG1-Fc. TUNEL and SpC immunostaining was pseudocolored white. H&E = hematoxylin and eosin; PBS = phosphate-buffered saline.
is another way by which Activin-A could contribute to pathophysiology. Up-regulation of TF in alveolar epithelial cells (30) and neutrophil-derived TF-mediated increased procoagulant activity has been previously described in patients with ARDS (36). Finally, the substantial inhibitory effect of Activin-A on expression of SpC, SpB, and SpA is of particular interest and provides an additional mechanism accounting for its pathogenic action in vivo. SpC-deficient animals develop pneumonitis and emphysema, and families with hereditary interstitial pulmonary disease carry SpC-gene mutations (37, 38). Significantly, analysis of BAL samples from patients with ARDS revealed a similar pattern of surfactant-protein expression, with SpC, SpB, and SpA being selectively down-regulated and SpD being unaffected (39).

Our findings may provide an explanation for the seemingly contradictory proinflammatory, antiinflammatory, and matrix-remodeling activities attributed to Activin-A. It is evident that in our study, some of the proinflammatory, antiinflammatory, and tissue-remodeling activities that have been ascribed to Activin-A by in vitro studies do occur in vivo. However, each of them manifests at different stages in the course of the evolving response, raising the possibility that some of them could share a “cause–effect” relationship, as previously demonstrated with the apoptotic and fibrotic responses induced by TGF-β overexpression in mouse lung (40). TGF-β1 was found to induce an early and transient wave of epithelial apoptosis, which, although repressed despite continuing TGF-β1 expression, was of key importance for the later development of fibrosis. Cytokines such as Activin-A or TGF-β can stimulate proinflammatory, antiinflammatory, or tissue-remodeling responses depending on the state of activation and differentiation of the target cells and the presence of other stimuli in the local microenvironment. Tissue damaging and proinflammatory effects of Activin-A could predominate during the acute phase of the response (a tradeoff between protection and collateral damage), whereas the antiinflammatory and matrix remodeling activities could reflect an ensuing Activin-A–induced protective or remodeling response.

An alternative explanation (which we cannot definitively exclude due to shortage of validated reagents for mouse follistatin protein quantitation) is that follistatin is eventually up-regulated at levels sufficient to neutralize Activin-A, and thus the antiinflammatory and tissue remodeling activities observed at the later stages of the response are not regulated directly by Activin-A. Analysis of phosphorylated Smad2 (pSmad2) levels in lung protein extracts demonstrated sustained up-regulation of pSmad2 levels in the Ad-ActA–treated group with kinetics similar to SpC levels from patients with ARDS. Conversely, the Ad-ActA and the LPS-driven ALI/ARDS–like models.

Even after tissue injury had been inflicted and the inflammatory response has developed, neutralization of Activin-A could still attenuate pathology. Therefore, our study strongly suggests that neutralization of Activin-A may reverse the complex and destructive cascade of ARDS and thus provide an additional therapeutic strategy for effective treatment of this syndrome.

**References**


