Thorax Online First, published on March 13, 2007 as 10.1136/thx.2006.063305

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# The Alveolar Epithelium can Initiate the Extrinsic Coagulation Cascade through Expression of Tissue Factor

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**ABSTRACT**
RATIONALE: The alveolar compartment is a pro-coagulant, anti-fibrinolytic environment in acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS). OBJECTIVE: To test the hypothesis that the alveolar epithelium can initiate intra-alveolar coagulation by expressing active tissue factor (TF). METHODS: Using an in vitro cell surface TF assay and TF ELISA we measured the activity and production of TF in cultured alveolar epithelial (A549) cells following exposure to cytomix (TNF-α, IL-1β, IFN-γ). TF gene transcription was measured by semi-quantitative RT-PCR. Immunohistochemistry for TF was done on lung sections of ARDS patients compared to controls. TF protein levels were measured by ELISA in undiluted pulmonary edema fluid from ALI/ARDS patients compared to control patients with hydrostatic pulmonary edema. MAIN RESULTS: TF activity, mRNA and protein levels increased in A549 cells after stimulation with cytomix. In addition, increased TF activity was measured on A549 cells following incubation with pulmonary edema fluid from ALI/ARDS patients. Immunohistochemistry for TF in human lung tissue from ARDS patients showed prominent TF staining in alveolar epithelial cells as well as intra-alveolar macrophages and hyaline membranes. Additionally, TF antigen levels (median of 37,113 (IQR 14,956-73,525) pg/mL in edema fluid vs 336 (IQR 165-669) pg/mL in plasma, $p<0.001$) and TF procoagulant activity were very high in the pulmonary edema fluid of ALI/ARDS patients compared to plasma, and higher plasma levels were associated with mortality. CONCLUSIONS: The alveolar epithelium is capable of modulating intra-alveolar coagulation through upregulation of TF following exposure to inflammatory stimuli and may contribute to intra-alveolar fibrin deposition in ARDS.

**KEYWORDS:** acute lung injury, acute respiratory distress syndrome, extrinsic coagulation cascade, hyaline membranes, fibrin deposition

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## INTRODUCTION

Intra-alveolar fibrin deposition is a pathologic hallmark of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) (1). Although the alveolar compartment in ALI/ARDS is procoagulant and antifibrinolytic (2, 3) the cellular and molecular mechanisms that modulate intra-alveolar fibrin deposition are incompletely understood. Previous studies have demonstrated that there is TF dependent procoagulant activity in bronchoalveolar lavage fluid in patients with ARDS (2, 4) however, comparison with a comparable control group (intubated patients with hydrostatic edema) was not done. The potential importance of the coagulation and fibrinolytic pathways in the alveolar space in ALI/ARDS needs further assessment. Clinical trials in ALI/ARDS have primarily targeted inflammation and have not focused on coagulation abnormalities. The recent success of an anticoagulant therapy in reducing mortality in severe sepsis (5) as well as progress in basic research (6) highlights the important pathogenetic role of coagulation abnormalities in inflammatory disorders.

Tissue factor (TF) is a 47 kDa transmembrane glycoprotein that is the most potent stimulator of the extrinsic coagulation cascade. Factor VIIa binds to TF on the cell surface and this complex binds to factor X converting it to the activated form Xa which leads to eventual thrombin formation and fibrin deposition. Idell *et al.* reported that TF activity correlated temporally with alveolar fibrin deposition in bronchoalveolar lavage fluid in a baboon model of acute lung injury (7) and that TF was an important contributor to the procoagulant activity in BAL fluid in patients with ALI/ARDS (2). Carraway *et al.* (8) demonstrated the central role of TF in sepsis-induced organ dysfunction, including lung injury, in an *E. coli* model of septic shock. In that study, baboons that were treated with site inactivated VIIa which blocks TF activity, had a dramatic reduction in physiologic and histologic lung injury as well as improved survival (8). Similar findings were reported using a blocking antibody for the TF-factor X interaction in the same model (9). Several studies have demonstrated the direct link between inflammation and tissue factor activation (10-12). Because of its central role in both fibrin generation and in linking inflammation and coagulation, TF may play a central role in the pathogenesis of ALI/ARDS.

Although both alveolar macrophages and alveolar epithelial cells have been identified as potential sources of alveolar TF, the specific time course and mechanism of alveolar epithelial TF production has not been well studied. Freshly isolated alveolar epithelial cells from rats can express TF but only one study showed a decrease in TF activity with PMA stimulation (13) while another demonstrated an increase in TF procoagulant activity (14). In addition, detailed information about the mechanism of TF expression by human alveolar epithelial cells is lacking. Therefore, we first hypothesized that active tissue factor would be present in the alveolar compartment of patients with ALI/ARDS as compared to patients with hydrostatic pulmonary edema and that the alveolar epithelium is a cellular source for soluble TF. Second, we hypothesized that the alveolar epithelium can express active TF and thus can actively modulate intra-alveolar coagulation and fibrin deposition.



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## METHODS:

**Collection of pulmonary edema fluid.** Samples of human pulmonary edema fluid and plasma were collected as previously described from intensive care unit patients at Moffitt Long and San Francisco General Hospitals from 1995-2001 (15). Briefly, mechanically ventilated patients with ALI/ARDS or controls with hydrostatic pulmonary edema were identified within six hours of endotracheal intubation using standard definitions (16). A standard tracheal suction catheter was inserted through the endotracheal tube and advanced slowly until resistance was met. Gentle suction was applied and a few milliliters of undiluted pulmonary edema fluid were aspirated. Samples were placed immediately on ice and centrifuged for 10 min at 3000 x g to remove any cellular or particulate matter. Supernatants were stored at -70°C until use. A simultaneous plasma sample was collected from each patient. The study was approved by the Committee on Human Research at the University of California San Francisco (UCSF). The Institutional Review Board at UCSF allowed a waiver of informed consent as the samples obtained were part of routine care and would otherwise have been discarded.

**Tissue factor enzyme linked immunosorbent assay.** A commercially available Enzyme Linked Immunosorbent Assay (ELISA) kit for TF (American Diagnostica, Stamford, CT) was used to measure protein concentrations of TF in human samples, cell lysates and supernatants according to the manufacturer's instructions. All measurements were done in duplicate.

**Effect of pulmonary edema fluid on clot formation in normal plasma.** Clot time was measured using a mechanical clot detection system (STart®4 Coagulometer, Diagnostica Stago, Asnieres, France). Briefly, pulmonary edema fluid was diluted 1:20 in PBS to a volume of 50 μL and incubated for 30 min at 37°C with either a blocking antibody to TF (American Diagnostica, Stamford, CT) or a species matched anti-human IgG antibody (Sigma, St. Louis, MO) at a dilution of 1:6. Samples were then incubated with 50 μL pooled normal plasma (Fisher Diagnostics, Middletown, VA) for 2 min. Clot time was determined as recalcification time following the addition of 8.3 mM calcium chloride. Measurements were done in duplicate.

**Immunohistochemistry study subjects.** Fresh lung tissue was obtained from 12 patients admitted to the adult medical intensive care units of the University of Utah Hospital, in Salt Lake City, between 1999 and 2002, who expired and underwent autopsy. The autopsy tissue was collected within 12 to 14 hours of death using standard anatomical pathology approaches optimized to preserve tissue architecture and antigen display. Autopsy tissue was obtained from 2 groups of 6 patients each. One group died with ALI or ARDS. Patients with ALI or ARDS were identified by the American European Consensus Conference definitions (16). The other group died without clinical or pathological evidence of pulmonary disease and served as controls. The autopsy protocol was approved by the Institutional Review Board at the University of Utah Health Sciences Center. The requirement for written informed consent was waived.

**Tissue collection and immunohistochemistry.** Two to three tissue cubes (>2 x 2 x 2 cm) were obtained from each of the three lobes of the right lung of each patient. The



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cubes were processed by standard methods (17). Briefly, the tissue cubes were immersion-fixed in 10% buffered neutral formalin (VWR, Inc., Media, PA) overnight at 4°C. Slices of the cubes were cut and processed for paraffin embedding for immunohistochemistry (6 μm section thickness).

For immunohistochemistry, antigen retrieval methods were used, followed by standard immunolabeling methods (17). Antigen retrieval was done, using 1x Citra solution (BioGenex, San Ramon, CA). Endogenous peroxidase was quenched by 3% hydrogen peroxide in phosphate buffered saline (PBS) for 20 min at room temperature. Between each of the steps, the tissue sections were rinsed in PBS. TF monoclonal antibody (American Diagnostica, Inc., Stamford, CT) was used at a dilution of 1:100 (overnight at 4°C). A standard ABC kit (Vector Laboratories Inc., Burlingame, CA) and DAB (Sigma-Aldrich Co., St. Louis, MO) were used to reveal tissue factor localization. All of the tissue sections were counterstained with hematoxylin, dehydrated through an ethanol series, cleared in AmeriClear, and covered by mounting medium and coverslip. Digital images were obtained using a Zeiss Axiophot (Jena, Germany) equipped with a digital camera. Brightness and contrast were adjusted to match background among the digital images; however, color adjustment and other image adjustments were not permitted.

For double immunofluorescence microscopy, deparaffinized lung sections were permeated with 1% Triton X-100, endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 20 min at room temperature, and antigen retrieval was performed, using 1x Glyca antigen retrieval solution (BioGenex, San Ramon, CA). Sections were blocked with 1% casein solution for 30 min at room temperature. Slides were incubated with a goat polyclonal anti-TF IgG antibody (1:500 and 1:1000 dilutions; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. Slides were then incubated with 1:250 anti-goat biotin conjugate (Vector Laboratories Inc., Burlingame, CA) for 60 min at room temperature, followed by 1:250 streptavadin-HRP (PerkinElmer, Boston, MA) for 60 min at room temperature. To visualize TF, the slides were incubated with 1:100 tyramide-FITC (PerkinElmer, Boston, MA) for 20 min at room temperature. Sections were then incubated with a rabbit polyclonal anti-pro-Surfactant Protein B antibody (Chemicon International, Temecula, CA) at a dilution of 1:250 for 60 min, then with 1:100 anti-rabbit-HRP conjugate (Vector Laboratories Inc., Burlingame, CA) for 60 min at room temperature, followed by tyramine-rhodamine (PerkinElmer, Boston, MA) for 20 min at room temperature. Sections were mounted with Vectorshield mounting medium containing DAPI nuclear stain (Vector Laboratories, Inc., Burlingame, CA). For all experiments, three negative controls were used: isotype- and species-matched irrelevant antibody (anti-insulin), omission of the primary antibody, and omission of the secondary antibody. In all cases, the negative controls demonstrated that the TF immunostaining was specific.

**Cell culture.** Alveolar epithelial cells (A549 cells, American Type Culture Collection, Manassas, VA) were grown in MEM (Cellgro, Herndon, VA) with 10% fetal bovine serum (Cellgro, Herndon, VA) and 10,000 units/mL each of penicillin and streptomycin (Sigma, St. Louis, MO). Small airway epithelial cells (SAEC) (Clonetics, San Diego, CA) were grown in complete small airway growth media (Clonetics). Both cell types were subcultured at the same density. For tissue factor activity assays cells were plated at



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1 x $10^4$ cells/well in a 96-well plate and maintained in culture until confluent at 5 days. For cell lysates and RNA extraction, cells were plated in a 24 well plate at 5 x $10^4$ cells/well and maintained in culture until confluence was reached at day 5. All experiments were performed on day 5 at confluence.

**Tissue factor activity assay.** Confluent monolayers of A549 cells were gently washed twice with 200 μL of serum-free MEM then incubated for varying lengths of time with serum-free MEM with or without the addition of various concentrations of cytomix. SAEC were washed twice with small airway basal medium and TF activity was measured in the same way. Cytomix is a mixture of equal amounts of TNF-α, IL-1β, and IFN-γ (R+D Systems, Minneapolis, MN). Following incubation, the cells were washed twice with 200 μL serum-free MEM. The cells were then incubated at 37°C for 60 min with 25 μL of 4nM factor VIIa and 25μL of 1 μM factor X. A factor Xa (factors VIIa, X, Xa Enzyme Research Laboratories, South Bend, IN) standard curve was prepared in a new 96 well plate. 100μL from each sample well was transferred to the new plate. After transfer, 5μL of 100mM EDTA was added to each well to stop the enzymatic reaction. Factor Xa was quantified by adding 100μL of the chromogenic substrate S-2222 (Chromogenix, Milano, Italy) to each well and incubating at room temperature for 15 min followed by measurement of OD 405. Control experiments were performed without the addition of factor VIIa and/or factor X. No TF activity was measurable in the absence of added VIIa or X (data not shown).

**Preparation of cell lysates.** A549 cells were grown to confluence in 24 well plates and exposed to cytomix or control medium. SAEC were grown in small airway growth medium. Following exposure the cells were incubated with 250 μL of lysis buffer (50 mM tris, 100 mM NaCl, 0.1% Triton X-100, pH 7.45) for 15 min at 37°C. The lysate was removed and placed in a clean microfuge tube and centrifuged at 3000g to remove cellular debris. The supernatants were stored at -70°C until assays performed.

**Cell viability assay.** Following the TF activity assay, cell viability was measured using a chromogenic electron coupling reagent (Promega, Madison, WI). Cell viability was compared between wells using control wells as a reference. Cytomix stimulation did not significantly alter cell viability (data not shown). TF activity assay results are expressed as "TF index" which controls for cell viability.

**Isolation of mRNA.** A549 cells were cultured in 24 well plates until confluent. Appropriate stimulus was applied in serum-free MEM to cells prior to mRNA isolation. In addition to cytomix, some cells were also treated with Actinomycin D at 5 μg/ml (Sigma-Aldrich, St. Louis, MO). For these experiments there were three treatment groups: untreated cells, cytomix alone, and actinomycin D plus cytomix (actinomycin was added 10 min prior to cytomix). Cells were then trypsinized and collected into cryotubes. Cells from three identical wells were pooled for each mRNA extraction. mRNA was extracted using a kit from Qiagen (Valencia, CA) according to manufacturer's instructions and stored immediately at -70°C. SAEC were washed with small airway basal medium and RNA was isolated in the same manner.



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**Real time PCR.** Semi-quantitative PCR was done using mRNA isolated from A549 cells or SAEC using the SYBRgreen method (Bio-Rad, Hercules, CA). Prior to the PCR reaction, cDNA was prepared using Superscript II RT (Invitrogen, Carlsbad, CA). All quantitative PCR reactions were run with a no template control for each primer set and a no cDNA control from each experimental condition. Both TF and β-actin primers were obtained from IDT (Coralville, IA) according to the specifications of Malarstig *et al* (18). Semi-quantitation was established using the ΔΔCt method. (19) Briefly, the Ct (confidence threshold) for each well was calculated by the Bio-Rad iCycler. For each experimental condition the Ct for β-actin was subtracted from the Ct for TF to generate the ΔCt. The difference between the ΔCt of the control experiment and the ΔCt of treated cells was calculated to give the ΔΔCt. Fold increase in mRNA was calculated by $2^{\Delta\Delta Ct}$ as a quantitative estimate.

**TF activity after exposure to human pulmonary edema fluid.** A549 alveolar epithelial-like cells were grown to confluence in 96-well plates. Confluent monolayers were washed with medium (RPMI) and incubated with pulmonary edema fluid or plasma from patients with ALI/ARDS at a 1:10 dilution with RPMI for 4 hours at 37°C and 5% $CO_2$. After repeated washing to remove any Xa present in the edema fluid, TF-dependent factor Xa activity on A549 epithelial cells was measured at 405 nm after incubation with purified Factor VIIa and Factor X at 2 hours using a specific chromogenic substrate (S-2765).

**Statistical Analysis.** Statistical analysis was done using SPSS software version 11.0 for Macintosh. In the *in vitro* experiments, differences between groups were assessed using a one-way analysis of variance (ANOVA) followed by Tukey test for post hoc analysis. Data from human samples were compared using the Student's T-test or Mann-Whitney U test depending on whether they were normally or non-normally distributed. Discrete patient data are presented as percentages, and comparisons between the groups were made with Pearson's chi-square test. When the assumptions of the chi-square were not met, a Fisher's exact test was used. For all analyses $p < 0.05$ was considered statistically significant.

## RESULTS

**TF levels in pulmonary edema fluid and plasma.** Patient characteristics are summarized in **Table 1**.

**Table 1.** Clinical characteristics of 54 patients with acute lung injury/acute respiratory distress syndrome (ALI/ARDS) and 28 patients with hydrostatic pulmonary edema from whom plasma and pulmonary edema fluid were obtained and 12 patients from whom lung tissue was obtained for immunohistochemistry.

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| Characteristic | Patients in whom plasma and edema fluid samples were obtained | | Patients in whom lung tissue was obtained for immunohistochemistry | |
|---|---|---|---|---|
| | **ALI/ARDS N=54** | **Hydrostatic N=28** | **ALI/ARDS N=6** | **Non-ALI/ARDS N=6** |
| Male gender | (30) 56% | (15) 54% | (4) 73% | (1) 16% |
| Caucasian | (36) 67% | (16) 58% | (6) 100% | (6) 100% |
| Age | 42 ± 16 | 54 ± 20[a] | 41 ± 16 | 53 ± 20 |
| Current smoker | (10) 19% | (8) 29% | (1) 16% | (1) 16% |
| Sepsis | (23) 43% | (0) 0% [b] | | |
| Lung injury score* | 3.0 ± 0.7 | 2.5 ± 0.7 | 3.6 ± 0.5 | 1.5 ± 1.2 |
| Tidal Volume (ml/kg) | 10.1 ± 2.9 | 10.5 ± 2.1 | | |
| SAPSII** | 48 ± 19 | 39 ± 15 | 62 ± 15 | 49 ± 18 |
| Ventilator-free days | 9 ± 11 | 17 ± 11 [c] | | |
| Hospital mortality | (29) 54% | (5) 19% [d] | (6) 100% | (6) 100% |

Data presented as either (n) % or mean ± SD
[a] p=0.009 vs ALI/ARDS; [b] p<0.001 vs ALI/ARDS; [c] p=0.006 vs ALI/ARDS; [d] p=0.001 vs ALI/ARDS
*Lung injury score (20)
**SAPSII, Simplified Acute Physiology Score II (21)



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TF levels in pulmonary edema fluid from 54 patients with ALI/ARDS were significantly higher than in 28 patients with hydrostatic pulmonary edema **(Figure 1A)**. In ALI/ARDS patients, TF levels in pulmonary edema fluid were more than 100-fold higher than simultaneous plasma samples (**Figure 1B)** with a median of 37,113 (IQR 14,956-73,525) pg/mL in edema fluid vs 336 (IQR 165-669) pg/mL in plasma, $p<0.001$. The very high levels of TF in edema fluid compared to simultaneous plasma samples strongly suggests an intra-alveolar source of TF. Higher plasma levels of TF were significantly associated with mortality, fewer ventilator-free days, the presence of disseminated intravascular coagulation (DIC), and the presence of sepsis in patients with ALI/ARDS. There were no differences in median tidal volume/kg between patients with hydrostatic edema compared to those with ARDS (10.4 mL/kg vs. 10.3 mL/kg $p=0.675$) or median PEEP levels at the time of edema fluid sampling between those with hydrostatic edema and those with ARDS (5.0 vs. 7.5, $p=0.072$). Minute ventilation was significantly higher in patients with ALI/ARDS (hydrostatic 13.5 L/min vs. ARDS 15.7 L/min, $p=0.01$) likely a reflection of increased dead space ventilation in ALI/ARDS (22). When ARDS patients with direct (aspiration and pneumonia) lung injury were compared with patients with indirect (sepsis, transfusion reaction, neurogenic edema) lung injury there were no differences in edema fluid or plasma tissue factor levels, severity of illness score, days of mechanical ventilation, ventilator-free days, ICU days, or mortality.

**Effect of pulmonary edema fluid on clot formation in normal plasma.** Clot time of normal plasma was significantly shorter in the presence of pulmonary edema fluid from patients with ALI/ARDS compared to pulmonary edema fluid from patients with hydrostatic edema **(Figure 1C)**. This difference was abolished by pre-incubation of pulmonary edema fluid samples with a blocking antibody to TF (**Figure 1D)**. Tissue factor protein levels were inversely associated with clot time in both ALI/ARDS and hydrostatic pulmonary edema (ALI/ARDS $r = -0.616$, $p <0.001$; hydrostatic $r= -0.757$, $p<0.001$ by Spearman's correlation).

**Tissue factor immunohistochemistry in human lung.** Six patients who died with ALI/ARDS and six control patients who died without pulmonary disease were studied. These patients were a different group from those from whom pulmonary edema fluid and plasma were obtained. Demographic and clinical characteristics of the patients are summarized in **Table 1**. Patients who died with ALI or ARDS tended to be younger, had more organ failures, and had a longer duration of assisted ventilation than patients who died without pulmonary disease, although the differences were not statistically significant. The most common causes of ALI or ARDS were sepsis (pulmonary or nonpulmonary) ($n = 3$) and pneumonia without sepsis ($n = 1$). The diagnosis of the control patients who died of nonpulmonary causes were cardiovascular or central nervous system disease. The immunohistochemical results shown in **Figure 2** were selected because they are typical for the 6 patients each who died with ALI/ARDS compared to those who died of nonpulmonary causes. TF immunostaining was displayed by cuboidal alveolar epithelial cells (**Figure 2 a**), squamous alveolar epithelial cells (**Figure 2 b**), neutrophils and alveolar macrophages in the airspaces, as well as within hyaline membranes in lung tissue sections from the patients who died with ALI/ARDS (**Figures 2 a and b**). These immunostaining patterns for TF were not seen in tissue sections from



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patients who died of nonpulmonary causes (**Figures 2 c**). Double immunofluorescence staining for TF (green) and pro-SP-B (red), an alveolar epithelial type II cell marker, demonstrated that TF co-localized (shown in yellow) to alveolar epithelial type II cells and alveolar epithelial type I cells in patients who died with ALI/ARDS (**Figures 2 f and g)**. Some yellow double immunofluorescent staining appears ambiguous with respect to the type of alveolar epithelial cell (**Figure 2 f**). That ambiguity appeared to be related to transition in cell phenotype from alveolar epithelial type II cells to type I cells during the reparative phase, which is typified by type II cell hyperplasia (**Figure 2 a**) that is followed by transition to type I cells as the injured lung tissue is repaired. In other areas along the alveolar membrane, TF was evident alone in alveolar epithelial type I cells (green; **Figures 2 f and g**). Minimal TF staining and co-localization was seen in the lungs of patients who died without ALI/ARDS (**Figure 2 h**).

**Tissue factor activity on A549 cells.** To determine whether alveolar epithelial cells can express active tissue factor, TF activity was measured in monolayers of A549 cells. Basally, A549 cells had almost no measurable TF activity **(Figure 3 A and C)**. However, TF activity was upregulated in a dose dependent fashion when the cells were exposed to increasing amounts of cytomix (TNF-α, IFN-γ, and IL-1β) (**Figure 3 A**). As little as 1 ng/mL of each cytokine resulted in a significant increase in TF activity on the cell surface. There was also a time dependent increase in TF activity when A549 cells were exposed to 20 ng/mL cytomix for increasing lengths of time (**Figure 3 C**). TF activity did not increase significantly until the 6 hour time point and peaked at 16 hours before declining slightly at 24 hours. (*$p<0.05$ compared to control using one-way ANOVA with post hoc Tukeys test).

**Tissue Factor protein concentrations in A549 cells.** There was a time and dose dependent increase in TF protein levels as measured by ELISA in cell lysates from A549 cells exposed to cytomix (**Figure 3 B and D**) that paralleled the increase in TF activity. (*$p<0.05$ compared to control using the one-way ANOVA with post hoc Tukey test). The rise in protein concentration in the cell lysates peaked at 6 hours.

**Tissue Factor gene expression.** Tissue factor mRNA was not present in A549 cells at baseline but was upregulated in a dose and time dependent manner after exposure to cytomix compared to β-actin. (*$p= 0.006$ vs 0 hrs, $p=0.025$ vs 1 hrs, $p=0.037$ vs 3 hrs, $p=0.024$ vs 24 hrs by the one-way ANOVA with post hoc Tukey test for multiple comparisons). **(Figure 4)** When cells were treated with 5 μg/mL Actinomycin D in addition to 20 ng/mL cytomix there was no increase in TF mRNA from 0 to 6 hours suggesting that changes in TF mRNA with cytomix stimulation are a result of new gene transcription and not secondary to changes in mRNA stability (data not shown).

**Effect of human pulmonary edema fluid on TF activity in A549 cells**. To determine the effect of a physiologic stimulus on TF activity in A549 cells, cells were exposed to pulmonary edema fluid or plasma from patients with ALI/ARDS. TF activity was increased on A549 cells following incubation with human pulmonary edema fluid from patients with ARDS but not following incubation with plasma samples from the same patients **(Figure 5)**. (*$p=0.003$ vs. plasma by Mann-Whitney U test).



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**Tissue Factor in Small Airway Epithelial Cells.** Primary isolates of human distal small airway epithelial cells were also assessed for TF gene and protein expression and cell surface TF activity. Under basal conditions in culture, these cells contain TF mRNA and TF protein, and have high levels of cell surface TF activity (data not shown) suggesting that they may also play a role in intra-alveolar fibrin deposition in lung injury.

## DISCUSSION

Tissue factor is the most potent initiator of the extrinsic coagulation cascade (23). The alveolar compartment in patients with ALI/ARDS is a procoagulant, anti-fibrinolytic environment and intra-alveolar generation of thrombin and fibrin likely has significant proinflammatory effects. We first tested whether TF was present in pulmonary edema fluid and plasma from patients with ALI/ARDS and compared them to a group of patients with hydrostatic pulmonary edema. TF protein levels were more than 100-fold greater in pulmonary edema fluid compared to plasma from the same patients. Plasma levels of TF in ALI/ARDS patients correlated with mortality, ventilator-free days, and the presence of sepsis suggesting that systemic activation of coagulation may be clinically important in acute lung injury. In addition to measuring protein levels, we also measured the ability of pulmonary edema fluid to stimulate clot formation in normal plasma. We found that edema fluid from both patients with hydrostatic edema or ALI/ARDS could stimulate clot formation but the time to clot formation was significantly shorter in ALI/ARDS confirming that the alveolar compartment in ALI/ARDS is pro-coagulant. In addition, the difference in clot time between hydrostatic edema and ALI/ARDS disappeared when TF activity in edema fluid was inhibited suggesting that the difference in clot time between hydrostatic edema and ALI/ARDS can be explained by the activity of tissue factor. There were no differences in tidal volume per kilogram, PEEP, or time to edema fluid sampling between hydrostatic and ALI/ARDS, suggesting that the differences seen were not due to differences in mechanical ventilation. Previous work has shown that there is an increase in TF dependent procoagulant activity in the alveolar compartment in ARDS (2, 4). However these studies were done on bronchoalveolar lavage fluid which has a variable degree of dilution making comparison between patients difficult. In addition, TF levels in the alveolar compartment in ARDS have never been compared to an appropriate control group such as patients with acute respiratory failure due to acute hydrostatic pulmonary hydrostatic edema. In the current study, direct aspiration of undiluted pulmonary edema fluid allowed direct comparison of levels and activity of TF in the alveolar compartment with other patients and simultaneous plasma samples, and enabled us to compare these measurements with a control group of critically ill patients with hydrostatic pulmonary edema.

Given the markedly elevated levels in pulmonary edema fluid compared to plasma, we hypothesized that there is an intra-alveolar source of TF. To determine the origin of intra-alveolar TF, we used immunohistochemistry to localize TF in human lung tissue. The alveolar epithelium stained positively for TF in ARDS but not in control lung, suggesting that it may be a source of intra-alveolar TF in patients with acute lung injury. This is in contrast to previous literature which reported TF staining of the alveolar epithelium in normal human lungs. (24, 25) However, these earlier studies made only a limited examination of TF staining in the lung, as part of studies of TF expression in all



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tissues; no colocalization was done to confirm alveolar epithelial expression. Our study is the first to carry out a detailed examination of the location of TF in the lung in patients with ALI/ARDS along with co-staining of the alveolar epithelium to identify the specific cellular type expressing TF. In vitro studies with A549 cells confirmed that the alveolar epithelium expresses active TF in response to a pro-inflammatory stimulus.

The alveolar epithelium, which covers a large surface area in the alveolar compartment, is known to participate in the modulation of coagulation through the protein C pathway (26) and also has other important functions in acute lung injury such as surfactant production and alveolar fluid clearance (27) by type II cells and sodium transport by type I cells (28) suggesting that both cell types play critical roles in the pathogenesis and resolution of acute lung injury. Little data exists regarding TF expression by cultured alveolar epithelial cells. One study reported a low level of TF activity in unstimulated A549 cells (29). Previous studies demonstrate that rat alveolar epithelial cells in culture produce functionally active TF (13, 14); however, as mentioned previously, those studies had contradicting results with one demonstrating a high level of TF activity in isolated alveolar epithelial cells that did not change with an inflammatory stimulus (14) and the other showing a decrease in TF activity following an inflammatory stimulus (13). In addition, there is little information on the time course of TF mRNA expression, protein expression and TF-dependent procoagulant activity in the alveolar epithelium. Our findings demonstrate that alveolar epithelial cells are capable of producing functional TF on the cell surface when exposed to pro-inflammatory cytokines or to pulmonary edema fluid aspirated from patients with ALI/ARDS. This finding suggests that the alveolar compartment contains mediators that are potent inducers of TF activity on the alveolar epithelium. The increase in TF activity on A549 cells results from increased TF mRNA transcription suggesting that at baseline the alveolar epithelium may not be capable of initiating coagulation but becomes procoagulant following exposure to an inflammatory stimulus. Our study is unique in that it provides detailed information about the time course of TF mRNA and protein production in alveolar epithelial cells and correlates this with cell surface Xa generating activity, findings that have not been previously reported. Additionally, the ability of the airway epithelium to modulate intra-alveolar coagulation through active TF expression was confirmed in primary small airway epithelial cells that contain TF mRNA and active cell surface TF basally. SAEC also play an important role in the extensive apoptosis seen in acute lung injury (30). In normal lungs taken at autopsy, low levels of TF were found in the alveolar epithelium; however, there was a dramatic increase in TF in the lungs of patients with ALI/ARDS. The TF was localized to alveolar epithelial cells, both type I and type II cells, as well as to macrophages and hyaline membranes.

Several experimental animal studies have demonstrated that TF expression in the lung is low at baseline but increases in response to systemic LPS administration (31, 32), hypoxia (33), or bleomycin lung injury (34). TF mRNA expression is increased in the lung and localizes to the alveolar epithelium in a rabbit model of endotoxemia (35). Our study demonstrates that alveolar epithelial TF expression increases in response to an inflammatory stimulus, a mechanism that may be important in the pathogenesis of acute lung injury. In a lethal baboon model of *E. coli* sepsis, systemic blockade of TF resulted in attenuated lung injury measured by physiologic parameters and histology (8, 9) highlighting the important role of TF in the development of lung injury. We have

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demonstrated TF immunostaining in alveolar epithelial cells and hyaline membranes in lung tissue from patients with ALI/ARDS, further supporting the hypothesis that the alveolar epithelium upregulates TF in response to inflammatory stimuli in the setting of ALI/ARDS and may be important in the pathogenesis of ALI/ARDS.

Several questions remain unanswered. While the high levels of TF found in human edema fluid compared to plasma are striking, the exact mechanism by which the TF is released into the alveolar compartment is unknown. It is clear that there is significant injury to and sloughing of the alveolar epithelium in patients with ALI/ARDS (36). Necrotic cells and apoptotic cells are a potential source for the very high intra-alveolar levels of TF in our patients. Alternatively, the TF could be cleaved from the cell surface or actively secreted. TF may also be released from alveolar macrophages.

There are some limitations to this study. First, we studied A549 cells, a human cancer cell line, which may not accurately represent in vivo responses, although these cells have been used in many cell and molecular investigations. However, the ability of the alveolar epithelium to express active TF was confirmed in primary isolates of SAEC which have TF mRNA and functional protein basally. In addition, the demonstration of TF in the alveolar epithelium in human lung tissue from ALI/ARDS patients gives credence to our *in vitro* experiments. Second, while our *in vitro* studies characterize the expression of TF in the alveolar epithelium, they do not explore the functional consequences of TF expression in ALI/ARDS. The ultimate consequences of this intense procoagulant stimulus in the alveolar compartment in patients with ALI/ARDS are unknown. There are several parallels between the intense tissue injury and coagulation abnormalities in the lung in ALI/ARDS and the systemic microvascular injury and thrombosis that develop in severe sepsis. Coagulation initiation and thrombus formation are central to the pathogenesis of sepsis (37) and modulation of coagulation with activated protein C can correct these derangements and improve patient outcome (5). It is possible that alveolar coagulation is similarly detrimental and production of thrombin, fibrin, and other coagulation proteins may promote injury and inflammation. Conversely, alveolar fibrin deposition may be important in localizing inflammation and preserving alveolar architecture in the acute phase when there is significant necrosis of the alveolar epithelium.

The current findings are important for several reasons. First, they further substantiate the alveolar epithelium as a source of intra-alveolar coagulation proteins that may be critical to the pathogenesis of ALI/ARDS and provide the first detailed immunohistochemical localization of TF in the alveolar compartment in ALI/ARDS. Second, by focusing on TF we have examined the initial steps of intra-alveolar fibrin deposition. By understanding the first critical steps that lead to intra-alveolar fibrin deposition and hyaline membrane formation we may be better able to modulate these end-points compared to modulating other coagulation proteins in the coagulation cascade. Finally, by identifying the alveolar epithelium as a significant source of coagulation proteins and characterizing in detail the increase in production of TF by an inflammatory stimulus, we are paving the way for epithelial-directed therapy that could be delivered by inhalation thereby avoiding the potential deleterious effects of systemic therapy and specifically targeting a key pathogenic mechanism in the development of ALI/ARDS.



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**ACKNOWLEDGEMENTS:** The authors thank Nancy Wickersham and Nimet Browne for their technical assistance.

Supported by National Institutes of Health grant HL70521 and HL081332 to LBW and HL51856 and HL74005 to MAM, an American Lung Association Biomedical Research Grant to LBW, and the Vanderbilt Physician Scientist Development Award to JAB.

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## FIGURE LEGENDS

**Figure 1. Comparison of plasma and pulmonary edema fluid tissue factor levels and clot time in patients with ALI/ARDS and control patients with hydrostatic pulmonary edema.** A and B: Boxplots of TF protein levels in edema fluid (A) and plasma (B) as measured by ELISA in patients with acute lung injury/acute respiratory distress syndrome (ALI/ARDS) or hydrostatic pulmonary edema (HYDRO). A: TF protein levels in pulmonary edema fluid (EF) were significantly higher in patients with ALI compared to hydrostatic edema (*p=0.012 by Mann-Whitney U test). B: TF protein levels in plasma were significantly higher in ALI/ARDS vs. HYDRO (**p=0.02 by Mann-Whitney U test). Note that edema fluid TF levels (panel A) are more than 100-fold higher than the simultaneous levels in the plasma (panel B) in both patient groups. C and D: Boxplots of clot time as measured by recalcification time of normal plasma mixed with pulmonary edema fluid from patients with ALI/ARDS or HYDRO in the absence (C) or presence (D) of a TF blocking antibody. Clot time was significantly longer in plasma mixed with edema fluid from patients with HYDRO compared to ALI/ARDS (C) (†p=0.006 by Mann-Whitney U test) and this difference was negated when TF activity was blocked (D) (p=0.095 by Mann-Whitney U test).

**Figure 2. Immunohistochemical localization of tissue factor in lung tissue sections from patients who died with ARDS [(+) ARDS] or without pulmonary disease [(-) ARDS].** TF immunolocalization is shown in *panels a-c*. Brown reaction product is visible in inflammatory cells (*) in the airspaces and along the epithelial cell lining (arrow) of the airspaces of the lung tissue section from a patient who died with ARDS (*panel a*). Cuboidal alveolar epithelial cells (arrow) are immunostained brown, indicating TF protein localization. In areas of thickened distal airspace walls of another patient, the squamous alveolar epithelial cells are distinctly immunostained brown (arrow; *panel b*). In contrast, immunolabeling of the occasional inflammatory cells in the airspaces is faint and patchy, and immunolabeling of alveolar epithelial cells is absent in the lung tissue from a patient who died of nonpulmonary disease (*panel c*). Immunostaining controls (omission of the primary antibody) are negative for lung tissue sections from a patient who died with ARDS (*panel d*) or with nonpulmonary disease (*panel e*). Double immunofluorescence staining was used to identify the cell type of alveolar epithelial cells that had TF immunostaining. TF protein was immunolabeled with green fluorochrome, whereas pro-SP-B was immunolabeled with red fluorochrome. Squamous alveolar epithelial cells are immunostained green in the tissue sections from the patients who died with ALI/ARDS (large arrows in *panels f and g*). Cuboidal alveolar epithelial cells are immunostained red (pro-SP-B) or yellow (co-localization of TF and pro-SP-B; short arrows in *panels f and g*) in the same tissue sections. By comparison, only cuboidal alveolar epithelial cells are immunostained red (pro-SP-B) or yellow (co-localization of TF and pro-SP-B; short arrows in *panel h*) in the tissue section from the patient who died from nonpulmonary disease. All panels are the same magnification (scale bar in *panels e and h* is 100 μm).

**Figure 3. Tissue factor activity and protein concentration in A549 cells increases in a dose dependent and time dependent manner.** Tissue factor (TF) activity in A549 cells



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increased in a dose dependent (panel A) and time dependent (panel C) manner in response to incubation with cytomix (TNF-α, IL-1β, IFN-γ). TF protein concentration as measured by ELISA increased in cell lysates under the same conditions. There is a dose dependent (panel B) and time dependent (panel D) increase in TF protein concentration in cell lysates (*$p<0.05$ compared to control in all experiments by one-way ANOVA with post hoc Tukey test). Data as mean ± SD.

**Figure 4. Increase in tissue factor gene expression in response to cytomix.** Tissue factor (TF) mRNA was measured using semiquantitative PCR and fold-change in mRNA was calculated using the $2^{\Delta\Delta Ct}$ method. When exposed to 20 ng cytomix for increasing lengths of time TF mRNA increased 34-fold at one hour and was still elevated above baseline (time 0) at 16 hours. *$p= 0.006$ vs 0 hrs, $p=0.025$ vs 1 hrs, $p=0.037$ vs 3 hrs, $p=0.024$ vs 24 hrs by the one-way ANOVA with post hoc Tukey test. Data as mean ± SD.

**Figure 5. Tissue factor activity is increased on A549 cells following exposure to pulmonary edema fluid but not plasma from patients with ALI/ARDS.** Boxplot comparing tissue factor (TF) activity on A549 cells after exposure to plasma or pulmonary edema fluid from patients with ALI/ARDS. Generation of factor Xa by A549 cells was measured with a chromogenic substrate and expressed as OD405 following incubation with edema fluid or plasma samples from 9 patients with ALI/ARDS (*$p=0.003$ by Mann Whitney U Test).



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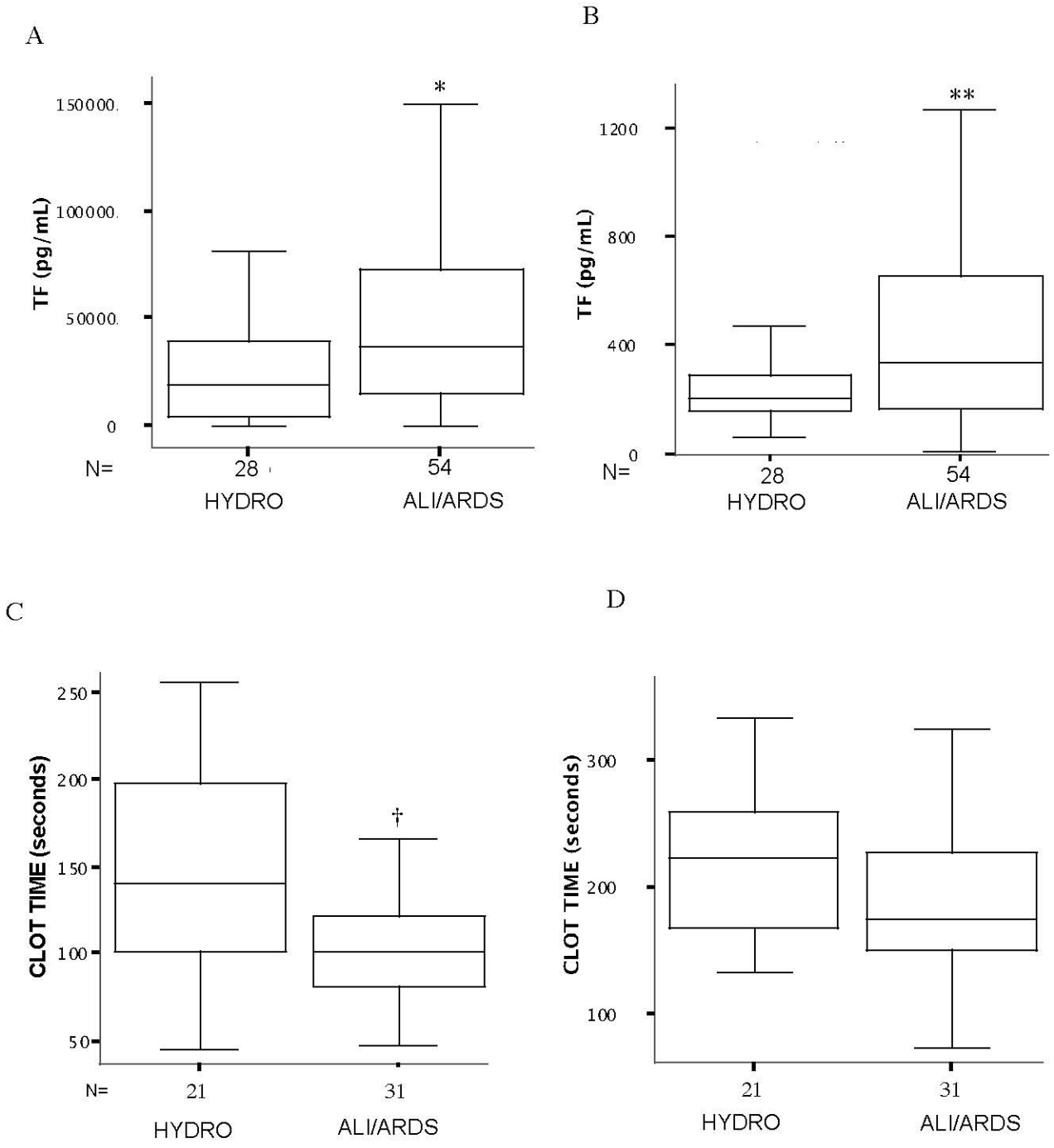


**Figure 1**

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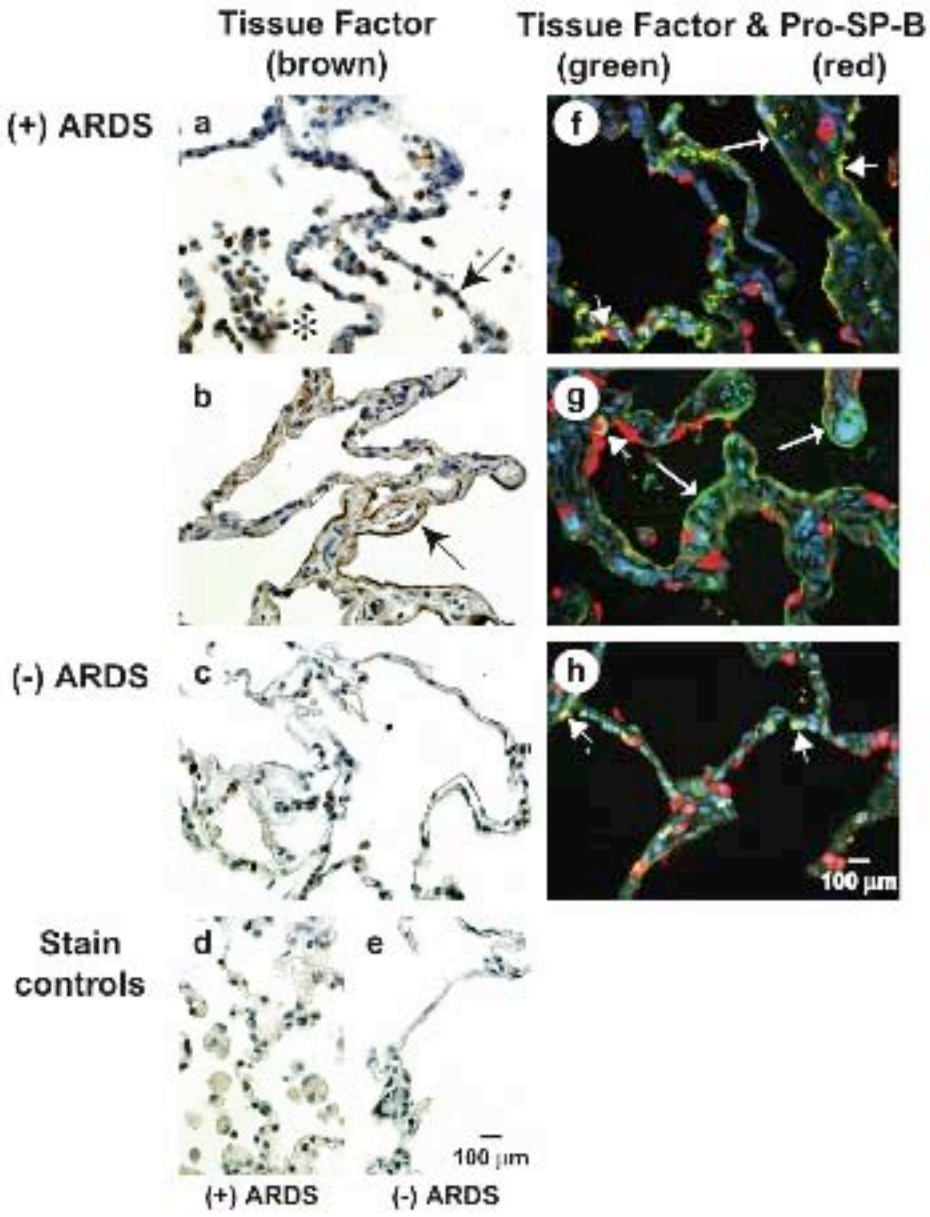


**Figure 2**

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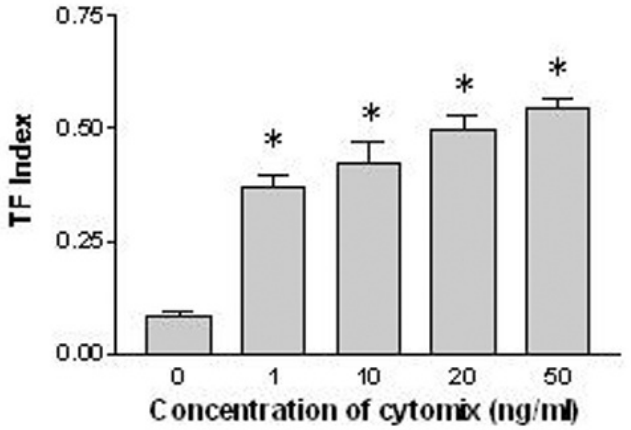


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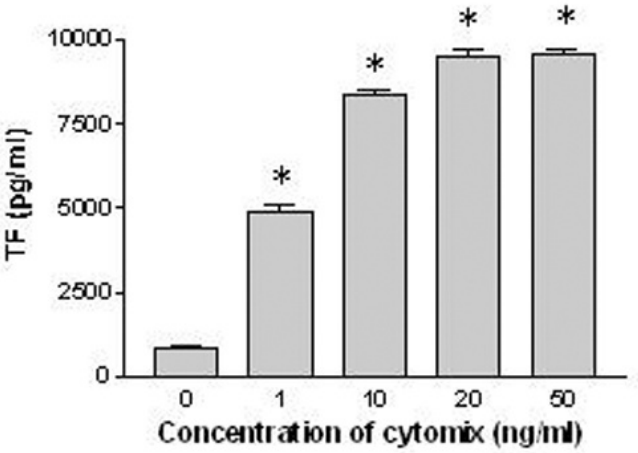


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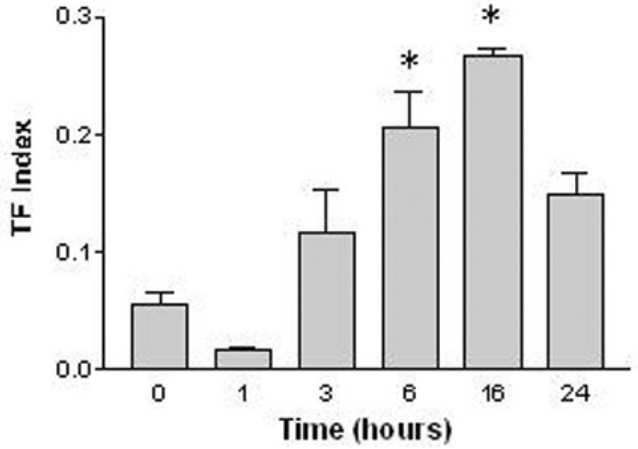


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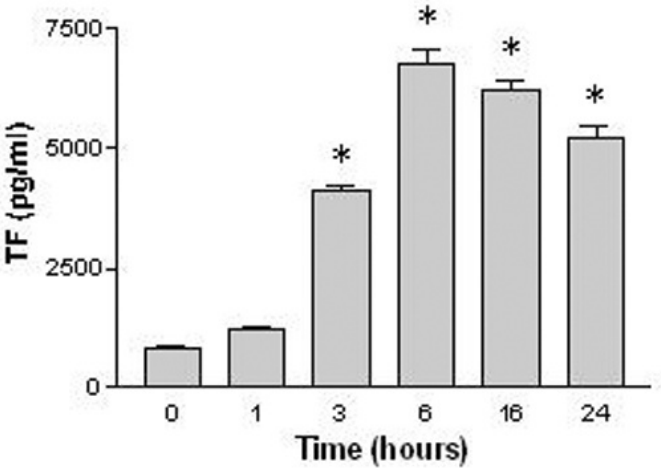


Figure 3

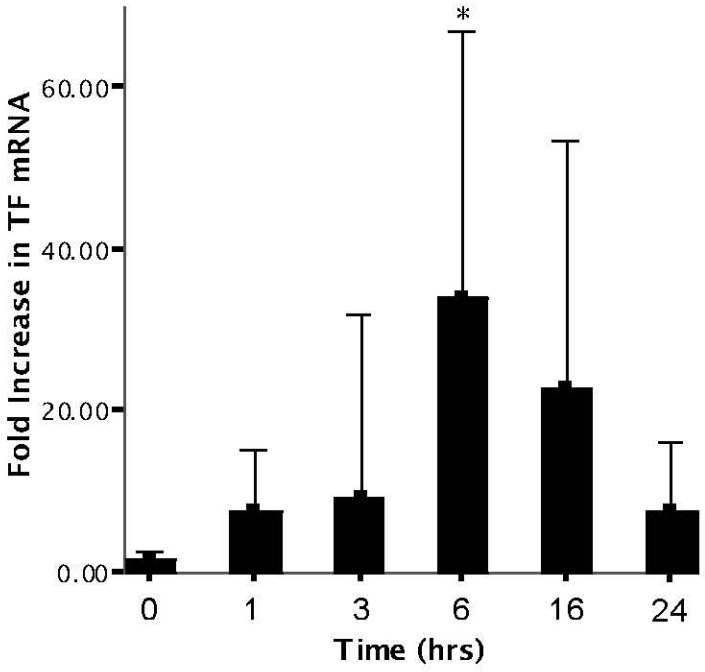


**Figure 4**

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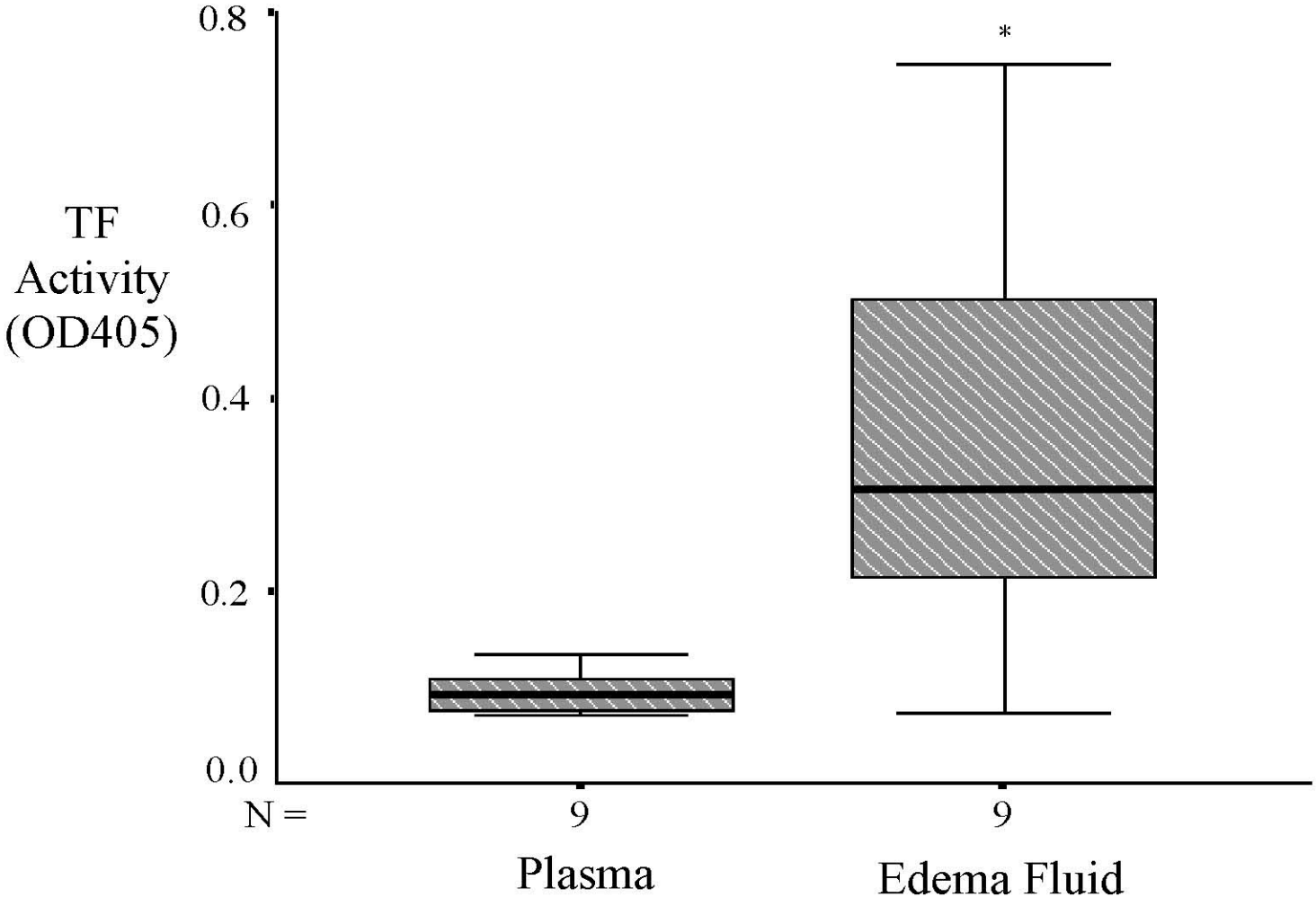


**Figure 5**

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