C5a and TNF-α Up-Regulate the Expression of Tissue Factor in Intra-Alveolar Neutrophils of Patients with the Acute Respiratory Distress Syndrome

Konstantinos Kambas,2* Maciej M. Markiewski,2§ Ioannis A. Pneumatikos,2† Stavros S. Rafail,* Vassiliki Theodorou, † Dimitrios Konstantonis, † Ioannis Kourtzelis,* Michael N. Doumas,§ Paola Magotti, ‡ Robert A. DeAngelis, ‡ John D. Lambris,3,4‡ and Konstantinos D. Ritis3,4*

Acute respiratory distress syndrome (ARDS) is characterized by the presence of fibrin-rich inflammatory exudates in the intra-alveolar spaces and the extensive migration of neutrophils into alveoli of the lungs. Tissue factor (TF)-dependent procoagulant properties of bronchoalveolar lavage fluid (BALF) obtained from ARDS patients favor fibrin deposition, and are likely the result of cross-talk between inflammatory mediators and hemostatic mechanisms. However, the regulation of these interactions remains elusive. Prompted by previous findings suggesting that neutrophils, under certain inflammatory conditions, can express functional TF, we investigated the contribution of intra-alveolar neutrophils to the procoagulant properties of BALF from patients with ARDS. Our results confirm that the procoagulant properties of BALF from ARDS patients are the result of TF induction, and further indicate that BALF neutrophils are a main source of TF in intra-alveolar fluid. We also found that BALF neutrophils in these patients express significantly higher levels of TF than peripheral blood neutrophils. These results suggest that the alveolar microenvironment contributes to TF induction in ARDS. Additional experiments indicated that the ability of BALF to induce TF expression in neutrophils from healthy donors can be abolished by inhibiting C5a or TNF-α signaling, suggesting a primary role for these inflammatory mediators in the up-regulation of TF in alveolar neutrophils in ARDS. This cross-talk between inflammatory mediators and the induction of TF expression in intra-alveolar neutrophils may be a potential target for novel therapeutic strategies to limit ARDS-associated disturbances of coagulation.

The acute respiratory distress syndrome (ARDS) is a severe and often life-threatening complication of several systemic disorders and direct injury to the lungs. It is associated with a high mortality rate (31–73%), primarily as a consequence of multiple organ failure and sepsis (1). The only approach proven to decrease mortality is mechanical ventilation with a low tidal volume (2). The formation of fibrin-rich exudates (hyaline membranes) in the lumen of lung alveoli is a morphological hallmark of ARDS (3). Intra-alveolar fibrin deposition occurring as a result of damage to the capillary endothelium or the alveolar epithelium significantly contributes to the pathogenesis of ARDS by decreasing surfactant activity, which favors alveolar collapse, and by decreasing alveolar fluid clearance (3, 4). The presence of fibrin in lung alveoli is accompanied by increased fibrin formation in the lung microvasculature, contributing to the loss of endothelial integrity and to thrombosis in the microcirculation (3–5). The injury to the pulmonary microcirculation resulting from inflammatory and thrombotic mechanisms contributes likely to the increase in dead space fraction that may be an independent predictor factor influencing ARDS-associated mortality (6). The intra- and extravascular deposition of fibrin indicates an increased procoagulant activity of blood in the lung microvasculature and alveolar fluid.

Bronchoalveolar lavage fluid (BALF) from ARDS patients has been shown to have procoagulant activity that is tissue factor (TF)-dependent (3, 7) and is more profound during the first 3 days following the clinical diagnosis of ARDS (7). TF triggers coagulation in vivo, and several inflammatory mediators, including complement anaphylatoxins and cytokines, up-regulate TF expression in circulating leukocytes, thereby increasing the thrombogenic activity of the blood (8, 9). Despite the extensive work that has been done to characterize the inflammation and fibrin deposition that occur in ARDS, the identity of the cellular elements and related inflammatory mediators that promote intra-alveolar coagulation are still elusive. Animal and clinical studies have indicated a pivotal role of neutrophils in the pathogenesis of acute lung injury.
Isolation of peripheral blood neutrophils

Peripheral blood neutrophils from all ARDS patients and from four healthy donors were separated by Histopaque-double gradient density centrifugation. The absolute number of neutrophils was adjusted to 2–3 × 10^6 cells/ml in PBS. Approximately one-quarter (6–8 × 10^5 cells in 250 μl PBS) was used for each stimulation or inhibition reaction. Cell purity (>98%), viability by trypan blue exclusion (>97%), and platelet contamination (<2 platelets/100 neutrophils) were assessed in all experiments. May-Grünewald-Giemsa staining did not reveal any platelets adhering to the neutrophils.

Modified prothrombin time (mPT) assay

Given that cell culture supernatants have been shown to have TF-dependent procoagulant activity (9, 22), we evaluated the procoagulant activities of BALF and neutrophil supernatants. In addition, our preliminary experiments showed that the procoagulant activity of neutrophil lysates assessed by recalibrated one-step clotting assays followed a similar pattern of changes to that observed for the procoagulant activity of neutrophil supernatants tested with a mPT assay. The differences in absolute values describing procoagulant activity of neutrophil lysates and supernatants were a result of the set of exogenous thrombin used in mPT assays. However, these differences do not affect the interpretation of obtained results. The presence of TF in supernatants is probably due to the presence of the soluble, spiked TF isoform (9) or TF micro-particles. The supernatants from neutrophils that had been incubated with BALF supernatants or various agents were isolated by centrifugation at 100 × g for 10 min. The coagulation activities (TF/FVIIIa binding activity) of cell supernatants used in the experiments were determined using a mPT assay. The absolute number of neutrophils was adjusted to 2–3 × 10^6 cells/ml. Neutrophils stimulated with BALF from patients with ARDS had been pretreated for 30 min with the selective nonpeptide antagonist of the anaphylatoxin receptor C3aR (SB-290152, 10 μg/ml final concentration) (25) or the selective C5aR antagonist AcF-[Opd-ChaWR (10 μg/ml final concentration) (26). In another group of studies, BALF from ARDS patients used for stimulation has been pretreated for 30 min with recombinant anti-human TF mAb (No. 4509; American Diagnostica) and isotype controls at concentrations of 10 μg/ml at room temperature (15). PT was then measured by the mPT method.

Stimulation and inhibition studies

Neutrophils from healthy individuals were incubated for 120 min at 37°C in a total volume of 250 μl of PBS, which contained various substances such as 1) serum from healthy individuals (30 μl); 2) serum from patients suffering from ARDS (50 μl); or 3) BALF from ARDS patients (40 μl). In addition to the set of exogenous thrombin used in mPT assays, we additionally tested with a mPT assay. The differences in absolute values describing procoagulant activity of neutrophil supernatants was in contrast to recalified one-step clotting assays. However, these differences do not affect the interpretation of obtained results. The presence of TF in supernatants is probably due to the presence of the soluble, spiked TF isoform (9) or TF micro-particles. The supernatants from neutrophils that had been incubated with BALF supernatants or various agents were isolated by centrifugation at 100 × g for 10 min. The coagulation activities (TF/FVIIIa binding activity) of cell supernatants used in the experiments were determined using a mPT assay. The absolute number of neutrophils was adjusted to 2–3 × 10^6 cells/ml. Neutrophils stimulated with BALF from patients with ARDS had been pretreated for 30 min with the selective nonpeptide antagonist of the anaphylatoxin receptor C3aR (SB-290152, 10 μM final concentration) (25) or the selective C5aR antagonist AcF-[Opd-ChaWR (10 μM final concentration) (26). In another group of studies, BALF from ARDS patients used for stimulation has been pretreated for 30 min with recombinant anti-human TF mAb (No. 4509; American Diagnostica) and isotype controls at concentrations of 10 μg/ml at room temperature (15). PT was then measured by the mPT method.

RNA extraction and relative quantitative real-time PCR analysis of TF mRNA synthesis

Total RNA was extracted, using the TRIZOL reagent (Invitrogen), according to the manufacturer’s instructions, from double-gradient purified BALF and peripheral blood neutrophils that had been collected at the same time as the BALF.

Tissue factor isoform-specific real-time PCR was performed to quantify the relative expression levels in the two TF isoforms with coagulant properties, full-length TF (referred to hereafter as TF) and alternative spiked TF (asTF) in the BALF-purified neutrophils according to the reference gene GAPDH and compared it to the normalized one of the patients’ peripheral blood neutrophils. The averages of the threshold values (C_T), as well as the DC_T and DDC_T values, were used for the 2^(-DDC_T) equation. Given the
Western blot analysis was performed as previously described (15). Other bars represent average mPT values of supernatants from normal neutrophils (H.N.) incubated with PBS (bar 1), serum from healthy individuals (H.S., bar 2), ARDS BALF (bar 5), and serum from ARDS patients (bar 7). Average mPT values ± SD of supernatants from normal neutrophils (H.N.) incubated ARDS BALF intermixed with anti-TF mAb are represented as bar 6. Mean values shown in this figure were obtained from three independent experiments performed with the use of BALF and sera from all ARDS patients included in the study (n = 7).

Western blot analysis
Cell lysates were prepared from ~2 × 10^6 cells using a lysis buffer containing 1% Triton X-100 in 150 mM NaCl/20 mM HEPES (pH 7.5) with protease inhibitors (Complete Protease Inhibitor tablets; Roche) and stored at −20°C. Western blot analysis was performed as previously described (15).

Table I. BALF cell characteristics

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Total BALF Cells</th>
<th>Analyzed BALF Volume (ml)</th>
<th>PMN (%)</th>
<th>Lymphocytes (%)</th>
<th>Multi-Nucleated Phagocytes (%)</th>
<th>Eosinophils (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4 × 10^6</td>
<td>18</td>
<td>76</td>
<td>6</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>15 × 10^6</td>
<td>15</td>
<td>92b</td>
<td>3</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>14 × 10^6</td>
<td>20</td>
<td>88bc</td>
<td>3</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>8 × 10^6</td>
<td>20</td>
<td>80</td>
<td>5</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>10 × 10^6</td>
<td>20</td>
<td>70</td>
<td>7</td>
<td>21</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>9 × 10^6</td>
<td>25</td>
<td>81bc</td>
<td>4</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>11 × 10^6</td>
<td>25</td>
<td>84c</td>
<td>4</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td><strong>Means</strong></td>
<td></td>
<td></td>
<td>81.57 ± 7.34</td>
<td>4.57 ± 1.51</td>
<td>12.14 ± 5.30</td>
<td>1.71 ± 1.50</td>
</tr>
</tbody>
</table>

a Double gradient centrifugation was also performed to obtain pure PMN population for quantitative real-time PCR (purity: 96.57 ± 1.27).

b FACS analysis was available in these BALF.

c May-Grünwald-Giemsa staining was used to identify BALF cells, and platelets were absent.

Luminex assay
Concentrations of growth factors, cytokines, and inflammatory mediators in BALF from ARDS patients were measured with the use of a Procarta cytokine profiling kit, according to the manufacturer’s instructions (Parnomics). After incubation with Ab-conjugated beads, detection Abs and streptavidin-PE complexes, samples were run on Bio-Plex instrument (Bio-Rad). Levels of the following growth factors, cytokines, and inflammatory mediators were evaluated: eotaxin, fibroblast growth factor-basic, G-CSF, GM-CSF, growth related gene product (GROα), IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40 and p70), IL-13, IL-17, IFN-γ inducible protein of 10kD, leptin, MCP-3, MIP-1α, MIP-1β, nerve growth factor, platelet derived growth factor BB, RANTES, TNF-α, TNF-β, and vascular endothelial growth factor.

C5a detection by ELISA
C5a levels in ARDS-BALFs were measured by ELISA designed “in house” and detecting only the C5a fragment of C5. Briefly, plates were coated with anti-C5a mAb (R&D Systems) then recombinant C5a (Sigma-Aldrich), which was used to generate a standard curve, and BALF samples were serially diluted on these plates. Polyclonal Abs against N- and C-terminal peptides of C5a (CGTLQKKEEEAKKYHSVVKK and CCVVASQLRANISHKDMQLGR, respectively) were used to detect bound C5a. Polyclonal anti-rabbit, HRP-conjugated IgG (Bio-Rad) was applied to detect C5a with Abs bound to Abs.

Statistical analysis
Data are expressed as means ± SD, except for C5a and cytokine concentrations, which are presented as medians and range. Statistical analyses
were performed using Student’s *t* and Mann-Whitney nonparametric (paired) tests to compare differences in means. The level of significance was set to *p* < 0.05. Data were processed using the STATISTICA version 5.0 (Statsoft) statistical program for Windows.

**Results**

**BALF from ARDS patients has strong procoagulant activity that is TF dependent**

To evaluate the procoagulant activity of BALF from ARDS patients, we measured the mPTs of BALF supernatants. Preliminary experiments showed that the procoagulant activity of BALF was dose dependent, reaching maximal levels when 150 μl of BALF was used for the assay; 40 μl of BALF had no effect on the mPTs, when compared with control values (data not shown). For additional experiments, a dose of 120 μl of BALF was used, because this amount of fluid induced significant shortening (*p* < 0.001) of the mPT (23.91 ± 1.30 s; Fig. 1, bar 3) when compared with the mPT of supernatants from neutrophils incubated with serum from healthy individuals (31.62 ± 0.45 s; Fig. 1, bar 2), or PBS alone (32.11 ± 0.58 s; Fig. 1, bar 1).

These assays further demonstrated that the procoagulant properties of BALF are dependent on the presence of functionally active TF, because preincubation of BALF supernatants with neutralizing anti-TF mAb completely abolished the procoagulant activity (31.95 ± 0.74 s; Fig. 1, bar 4).

**Neutrophils accumulating in the lumen of pulmonary alveoli in ARDS patients are a major source of TF**

Given that we have recently shown that neutrophils produce functionally active TF in patients with antiphospholipid syndrome (15) and that these cells constitute the major cellular population in the BALF from ARDS patients (88%), we have performed detailed experiments to determine the procoagulant activity of these cells.

**FIGURE 3.** The 2−DDC** data analysis. Relative quantification of TF and asTF in circulating (*n* = 7) and BALF purified PMNs (*n* = 6). Relative expression (indicated by bars) was based on the average DC T values of the target gene (TF or asTF) and GAPDH (DC T of TF BALF-purified neutrophils 5.652 ± 0.85 vs 8.798 ± 0.94 of TF blood PMNs and DC T of asTF BALF-purified neutrophils 11.435 ± 1.10 vs 16.054 ± 1.97 of asTF blood PMNs).
FIGURE 4. BALF C5a or TNF-α inhibition abolishes TF-dependent procoagulant activity. A, The median and range of concentrations for C5a, TNF-α, and IL-6 (pg/ml) in BALF from ARDS patients (n = 7). B, mPT values of supernatants from neutrophils (H.N.) originating from healthy volunteers and stimulated with ARDS serum (bar 1), ARDS BALF alone (bar 2), ARDS BALF after pretreatment with C5aR antagonist (C5aRA) (bar 3) or with addition of anti-TNF-α Ab (bar 4). Bars represent the average of mPT values ± SD from experiments using BALF and sera from ARDS patients (n = 7). Three independent experiments were performed and representative results are shown. C, TF expression in healthy neutrophils (H.N.) incubated with sera from an ARDS patient (I; Ia: negative control anti-CD19 staining; and Ib: anti-TF staining), ARDS BALF alone (II), ARDS BALF after pretreatment of H.N. with C5aRA (III), or with addition of anti-TNF-α Ab (IV). Images show immunostaining with the use of anti-TF Ab. Binding of anti-TF Ab was visualized by APAAP method (magnification × 1000). Three independent experiments using sera and BALF from ARDS patients were performed (n = 7) and representative examples are shown. D, Representative FACS analysis of H.N. (n = 4) identified by forward- and side-scatter characteristics, before and after stimulation of cells with BALF, as well as after C5aR and TNF-α inhibition studies. E, Representative examples of western blot analyses (n = 7 for each individual experiment). Three independent experiments were performed. Lanes correspond to immunostaining and mPT analyses. Lane I: H.N. incubated with ARDS serum; Lane II: H.N. stimulated with 40 μl BALF; Lane III: H.N. pretreated with C5aRA and stimulated with BALF; Lane IV: H.N. incubated with BALF and anti-TNF-α. Lane V: TF expression from total cell extracts from BALF with a neutrophil purity of 84%.
BALF from ARDS patients, as shown previously (16) and by our current study (Table I), we hypothesized that neutrophils also express TF within the alveoli of ARDS-affected lungs. To test this hypothesis, we characterized the expression of TF in various cell types from the BALF of ARDS patients. Immunostaining of smears prepared from this fluid showed that >85% of neutrophils expressed TF in all seven analyzed samples, whereas no expression or only weak staining was observed on peripheral blood neutrophils from the same patients (Fig. 2A). Multinucleated phagocytes that are generated by fusion of alveolar macrophages in response to infections showed positive cytoplasmic staining, mainly in two of the seven patients’ samples (Fig. 2B, I). Alveolar macrophages exhibited a diffuse cytoplasmic staining, whereas pneumocytes (Fig. 2B, II), eosinophils, and lymphocytes were negative (data not shown). The TF expression in BALF neutrophils was notably higher than in patients’ peripheral blood PMNs, and this observation was corroborated by flow cytometry analysis (Fig. 2C).

To confirm that the TF visualized in BALF neutrophils by immunostaining and flow cytometry was indeed produced by these cells, real-time PCR analysis was performed using RNA extracted from purified BALF neutrophils (purity 96.57 ± 1.27; Table I) and patients’ peripheral blood neutrophils. This analysis showed that expression of TF mRNA was significantly higher in purified BALF neutrophils than in peripheral blood neutrophils from the same patients, with αsTF being the dominant isoform (Fig. 3).

**Mediators present in alveolar fluid in patients with ARDS induce the expression of TF in neutrophils**

The up-regulation of functionally active TF in alveolar neutrophils and the lack of such up-regulation in peripheral blood neutrophils suggest that these cells are stimulated to produce TF locally in the lumen of alveoli, or when they are crossing the endothelial barrier or are present in the lung microcirculation. To test the first possibility, freshly isolated neutrophils from healthy donors were incubated with 40 μl of BALF supernatant from ARDS patients. This dose was selected because, as shown by our previous experiments, this amount of BALF did not result in a shortening of the mPT. Therefore, the residual amount of TF originating from the BALF used for neutrophil stimulation was not expected to interfere with the mPT assay, which used supernatants from activated neutrophils after stimulation. Indeed, we observed that supernatants isolated from BALF-activated neutrophils exerted procoagulant properties, inducing a significant shortening (p < 0.001) of the mPT to 24.13 ± 1.31 s (Fig. 1, bar 5) when compared with controls (Fig. 1, bars 1 and 2). This procoagulant activity was TF-dependent, because addition of anti-TF Ab to the supernatants from the activated neutrophils abrogated the procoagulant effect (31.73 ± 0.37 s; Fig. 1, bar 6).

Conversely, sera from ARDS patients did not induce significant procoagulant activity in neutrophils isolated from healthy individuals (29.69 ± 1.64 s; Fig. 1, bar 7). These results suggest that mediators that can induce the expression of functionally active TF in neutrophils are present in the alveolar fluid of ARDS patients.

**C5a and TNF-α stimulate alveolar neutrophils to produce TF**

Inflammatory mediators, including complement anaphylatoxins and cytokines, enhance the thrombogenicity of blood by up-regulating TF in circulating leukocytes and endothelial cells (31). In addition, recent studies in human neutrophils (15) and animal models (32) have demonstrated that C5a stimulates neutrophils to produce TF. Importantly, acute lung injury in mice is C5a dependent (19). Furthermore, C5a (18), along with TNF-α (33) and IL-6 (33), accumulates in BALF of ARDS patients. Our studies confirmed that C5a, TNF-α, and IL-6 are indeed detectable in BALF from ARDS patients (Fig. 4A). Therefore, we hypothesized that complement anaphylatoxins and/or cytokines present in alveolar exudate might stimulate neutrophils to produce TF in the course of ARDS.

To test this hypothesis, we determined the procoagulant activity of supernatants from healthy volunteers’ neutrophils stimulated by BALF from ARDS patients, before and after treatment of the neutrophils to produce pharmacological blockade of C3a, C5a, TNF-α, or IL-6. The mPT analysis showed that the blockade of C5a or TNF-α signaling inhibited BALF-induced procoagulant activity of neutrophil supernatants and resulted in significant increase (p < 0.001) of the mPT values to 31.91 ± 0.64 s (Fig. 4B, bar 3) and 30.43 ± 0.52 s (Fig. 4B, bar 4), respectively, when compared with the mPT of neutrophils from healthy volunteers incubated with BALF (24.13 ± 1.31 s; Fig. 4B, bar 2). Thus, the blockade of C5a or TNF-α signaling returned the mPT to values for control cells stimulated with ARDS serum (29.69 ± 1.64 s; Fig. 4B, bar 1). In contrast, a lack of C3a or IL-6 signaling did not influence the mPT values of supernatants from BALF-stimulated neutrophils (data not shown). The loss of BALF-induced procoagulant activity as a result of the blockade of C5a or TNF-α signaling was associated with the loss of TF expression, as demonstrated by immunostaining, flow cytometry, and western blot analyses (Fig. 4, C–E).

**BALF from ARDS patients contains numerous chemokines facilitating migration of neutrophils into pulmonary alveoli**

To further characterize the inflammatory microenvironment in pulmonary alveoli of ARDS-affected lungs we assessed levels of various cytokines, growth factors, and inflammatory mediators in ARDS BALF from all patients included in the study. The concentrations of factors that were detectable in at least four out of seven BALF samples are shown (Fig. 5). Remarkably, we observed that various chemokines that are known to facilitate migration of neutrophils into inflamed tissues or to be expressed by neutrophils, such as IL-8, GROα, MIP-1α, MIP-1β, and IP-10, are present in ARDS BALF in high concentrations. In addition, several mediators that induce the expression of chemokines in neutrophils were also detectable in ARDS BALF, including TNF-α (Fig. 4), IL-1β, and GM-CSF (Fig. 5).
Discussion

In the present study, we have established that alveolar neutrophils of ARDS patients up-regulate TF in lung tissue, contributing to the abnormal deposition of fibrin within pulmonary alveoli that is associated with ARDS. We have further shown that this expression is essentially regulated by inflammatory mediators (C5a and TNF-α, but not C3a or IL-6) acting in the alveolar microenvironment. Although the presence and activity of TF in ARDS BALF have been well-documented (7), the source of this TF had not been clearly established. Our findings demonstrate a local and pivotal role for neutrophils in TF expression and may provide a link between these cells and the procoagulant activity seen in BALF from ARDS patients. Our results indicate that only BALF neutrophils, and not circulating neutrophils, express high levels of functionally active TF, which exerts its effects at the local level, in the microvasculature, and alveoli of the lung.

The findings presented here provide an explanation for, and underscore, the critical importance of neutrophils in the progression of ARDS, which has already been well-documented. Studies using various experimental models in animals have indicated that the severity of the ALI/ARDS course depends on the number of activated neutrophils infiltrating the lung (34). Although ARDS has been described in neutropenic patients (35), these findings do not exclude the central role of neutrophils in ARDS pathophysiology, because the recovery of patients from neutropenia was associated with deterioration of pulmonary function (36). Attenuation of neutrophil accumulation in the lungs, by using molecules inhibiting neutrophil adhesion, decreased the severity of lung injury (37). In addition, data from animal studies indicate that neutrophil depletion markedly attenuates the severity of endotoxia-induced ALI (38, 39).

Although it had been known for some time that C5a up-regulated TF expression in endothelial cells and monocytes (31), the idea that TF could be produced by neutrophils activated by inflammatory mediators was controversial. Only recent studies have demonstrated that neutrophils can produce TF when they are activated by the complement anaphylatoxin C5a (15, 32). Our experiments indicate that such activation can occur locally in the lungs of ARDS patients. It is well recognized that the functions of complement and inflammatory mediators are highly context dependent. Under various pathophysiological conditions these mediators can exert different or even opposite effects (40). Therefore, despite the fact that C5a has been shown to stimulate neutrophils to produce TF in patients with antiphospholipid syndrome, this study provides a novel and significant insight into ARDS-associated coagulopathy.

Given that complement is activated in the course of ALI/ARDS (18, 19) and that neutrophils accumulate in ARDS-affected lungs (16), it was reasonable to hypothesize that these cells would produce TF as a result of their activation by C5a. Importantly, the inhibition of C5a has also resulted in attenuation of lung injury (41, 42).

Our data confirm previous reports that BALF obtained from ARDS patients has procoagulant properties that are TF dependent (7), and we have further demonstrated by immunostaining and flow cytometry that intra-alveolar neutrophils express high amounts of TF. Real-time PCR analysis confirmed that the neutrophils present in BALF produce TF, indicating that the neutrophils are indeed a main source of TF, and they do not merely acquire this protein from microparticles released by other cells. Also, our demonstration that BALFs from ARDS patients are capable of inducing procoagulant activity in neutrophils from healthy donors suggests that factors inducing TF expression in intra-alveolar neutrophils in ARDS patients are present in the intra-alveolar fluid. The fact that neutrophils from the plasma of ARDS patients lacked this capacity strongly suggests that neutrophil stimulation occurs locally within the lumen of the pulmonary alveoli or the lung microcirculation. In addition to C5a (16, 18), intra-alveolar fluid in ARDS patients is rich in other inflammatory mediators, such as those already mentioned here: TNF-α, IL-1, IL-6, and IL-8, all of which are present in the lungs at higher concentrations than in plasma (33, 43). The high levels of these cytokines in BALF have been associated with increased mortality in patients with ARDS (44). It has also been shown in animal experimental models of ALI/ARDS that TNF-α suppression can decrease lung injury (45). However, the recruitment of neutrophils to the ARDS-affected lungs was reported to be TNF-α independent (45). Like C5a, TNF-α acts as a strong stimulator of TF expression in leukocytes and endothelial cells (9). Therefore, we also investigated whether TNF-α-mediated signaling contributes to the production of TF in pulmonary alveoli in ARDS patients.

A role for both TNF-α and C5a in ARDS-associated up-regulation of TF was further supported by our demonstration that pharmacological blockade of TNF-α and C5a signaling from healthy volunteers was able to significantly diminish the BALF-induced procoagulant activity of these otherwise normal cells and cause a concomitant loss of TF expression. These results indicate that C5a and TNF-α signaling contributes to the induction of TF expression in neutrophils accumulating in the alveoli of lungs affected by ARDS. However, further studies are required to establish conclusively whether C5a and TNF-α both stimulate neutrophils directly or whether C5a acts only as an upstream regulator of TNF-α.

Our study has also shown that, besides cytokines that are already known to be present in the intra-alveolar environment of ARDS affected lungs, BALF contains various other mediators not previously reported as being associated with ARDS pathology. Although the levels of these cytokines were highly variable, which could be expected in samples obtained from patients, the striking hallmark of the overall BALF cytokine profile was the presence of various chemokines associated with neutrophil migration. Importantly, several of these chemokines can be produced by neutrophils themselves and further amplify accumulation of these cells in inflamed tissues (46).

The procoagulant properties of intra-alveolar fluid have important consequences for ARDS pathophysiology. The presence of functionally active TF in intra-alveolar fluid favors the formation of hyaline membranes that limit the area of active gas exchange, affect surfactant activity, and contribute to vascular congestion (6). In addition, the subsequent fibrosis of hyaline membranes leads to irreversible changes that cannot be resolved. Recent investigations showed also that the use of anti-TF factor Ab significantly ameliorated intestinal ischemia reperfusion induced acute lung injury in mice (47). Interestingly, studies suggesting the role of TNF-α-mediated inflammation (48) and neutrophils (49) in bleomycin-induced pulmonary fibrosis connect our findings to this particular aspect of ARDS pathophysiology. All of these consequences of TF activity contribute to the development of severe respiratory insufficiency in ARDS patients. Therefore, efforts to limit intra-alveolar fibrin deposition in the course of ARDS have particular significance for improving the effects of ARDS therapy.

The important conclusion of our studies is that in ARDS patients, neutrophils are activated locally in the lung. Therefore, it is highly desirable to design therapies that may affect inflammatory mediators in the immediate alveolar microenvironment. Our findings therefore confirm previous studies in animal models of ALI/ARDS, pointing to the potential usefulness of local blockade of C5a or TNF-α signaling in improving the course of lung injury (41, 42, 50), and they provide a mechanism to explain how neutrophils contribute to the abnormal procoagulant activity associated with this potentially life-threatening condition.
Acknowledgments
We thank Dr. D. McClellan for excellent editorial assistance.

Disclosures
The authors have no financial conflict of interest.

References