

# C5a and TNF- $\alpha$ Up-Regulate the Expression of Tissue Factor in Intra-Alveolar Neutrophils of Patients with the Acute Respiratory Distress Syndrome<sup>1</sup>

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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- $\alpha$  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 Journal of Immunology*, 2008, 180: 7368–7375.

The acute respiratory distress syndrome (ARDS)<sup>5</sup> 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 (7) 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

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<sup>5</sup> Abbreviations used in this paper: ARDS, acute respiratory distress syndrome; BALF, bronchoalveolar lavage fluid; TF, tissue factor; PMN, polymorphonuclear cell; ALI, acute lung injury; APAAP, alkaline-phosphatase anti-alkaline-phosphatase; mPT, modified prothrombin time; asTF, alternative spliced TF; GRO $\alpha$ , growth related gene product.

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(ALI) and ARDS (10–12). Histological analyses of autopsy specimens have revealed a pronounced accumulation of polymorphonuclear cells (PMN) in the injured pulmonary alveoli (13) and in the interstitial tissue of the lungs affected by ARDS (14).

Our recent work has demonstrated that C5a induces TF expression in circulating blood neutrophils in patients with antiphospholipid syndrome (15). Because neutrophils constitute the major cellular population that is present in BALF and in autopsy samples from patients with ALI/ARDS (16, 17) and complement components are involved in the pathogenesis of these disorders (18, 19), we hypothesized that the procoagulant properties of ARDS-BALF are complement-dependent. In particular, we have postulated that C5a, by causing the up-regulation of TF expression in intra-alveolar neutrophils, enhances local fibrin generation, thereby contributing to the formation of hyaline membranes.

In this work, we have shown that neutrophils, which are present in intra-alveolar exudates, play a significant role in the TF-dependent procoagulant activity of BALF from ARDS patients. Local production of TF by neutrophils in lung alveoli was C5a- and TNF- $\alpha$ -dependent, because pharmacological blockade of C5a or TNF- $\alpha$  signaling resulted in the abrogation of BALF-induced TF expression in neutrophils collected from healthy individuals. Thus, our study reveals a novel role for neutrophils stimulated by C5a and TNF- $\alpha$  to produce TF locally in the pulmonary alveoli, in the formation of hyaline membranes in patients with ARDS.

## Materials and Methods

### Patient population

All patients included in this study were recruited from the Intensive Care Unit of Academic Hospital in Alexandroupolis, Greece. The Institutional Review Board approved the protocol, and all procedures were in compliance with institutional guidelines. Informed consent was obtained from the closest relatives.

Seven consecutive nonsmoking patients (mean age:  $55 \pm 17$  years) participated in the study. Patients fulfilled the criteria of the American-European Consensus Conference on ARDS (20) for ARDS diagnosis, and all of them were mechanically ventilated according to the ARDSnet protocol (2). Ventilator setting adjustments were applied according to individual patient's requirements. Volume substitution and vasoactive or inotropic drugs were administered as required, and antibiotic therapy was guided by microbiological findings. Extrapulmonary infection with sepsis was considered as the possible etiology in five, and severe multitrauma in two individuals included in this study. All patients underwent flexible bronchoscopy for BALF collection during the first 3 days after ARDS diagnosis because temporal changes in ARDS BALF indicate that this time point is characterized by enhanced procoagulant activity (7).

### Preparation of BALF (BALF collection, isolation of BALF cells, and supernatants)

BALF collection was performed as described in a previous study (21). Six 20-ml aliquots of sterile 0.9% saline were infused and removed by gentle suction (recovery 20–30%). The first aspirated fluid reflected a bronchial sample and was used for microbiological screening, while the remaining aspirates were collected separately in sterile tubes. The contents of various tubes originating from a single patient were mixed to obtain a representative cellular population. The BALF was then filtered through sterile gauze and immediately used in the study. Approximately 15–25 ml of the filtered BALF was immediately centrifuged for 15 min at  $800 \times g$  at  $4^\circ\text{C}$  to isolate BALF supernatants for functional assays and stimulation/inhibition assays. The remaining pellet of BALF cells was washed in PBS, re-diluted in 1 ml PBS, and used for cell counts and cell viability assessment by trypan blue staining. Another portion of the cell pellet was immediately used for FACS analysis ( $n = 4$ ), and the remaining cells were smeared on glass slides for May-Grünwald-Giemsa staining and alkaline-phosphatase anti-alkaline-phosphatase (APAAP) immunostaining and then kept at  $-20^\circ\text{C}$  till use, or stored as pellets at  $-20^\circ\text{C}$  for later experiments. In six patients, an additional neutrophil separation was also performed using Histopaque 1077/1119 double-gradient density centrifugation (Histopaque; Sigma-Aldrich), according to the manufacturer's instructions, to obtain highly purified neutrophils (>95%) for higher accuracy in semiquantitative real-time PCR analysis.

### 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  $2\text{--}3 \times 10^6$  cells/ml in PBS. Approximately one-quarter ( $\sim 6\text{--}8 \times 10^5$  cells in 250  $\mu\text{l}$  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-Grünwald-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 recalcified 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 use of exogenous thromboplastin in mPT in contrast to recalcified 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, spliced TF isoform (9) or TF micro-particles. The supernatants from neutrophils that had been incubated with BALF supernatants or/and various agents were isolated by centrifugation at  $1000 \times g$  for 10 min. The coagulation activities (TF/FVIIa binding activity) of the cell supernatants were determined using an mPT assay as previously described (15). This assay is a modification of the classical PT assay, and its principles are also similar with recalcified one-step clotting assay (23, 24). In addition, the mPT assay offers a fast, reliable, nonexpensive, and easily-reproducible first screening test of procoagulant activity. To verify that the thromboplastic activity was due to TF, supernatants were incubated for 30 min with a specific mouse anti-human TF mAb (No. 4509; American Diagnostica) and isotype controls at concentrations of 10  $\mu\text{g}/\text{ml}$ , at room temperature (15). PT was then measured by the mPT method.

### Stimulation and inhibition studies

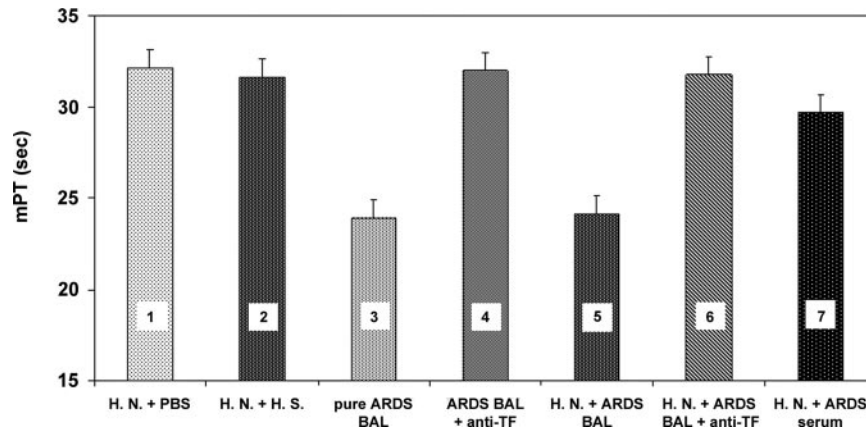
Neutrophils from healthy individuals were incubated for 120 min at  $37^\circ\text{C}$  in a total volume of 250  $\mu\text{l}$  of PBS, which contained various substances such as 1) serum from healthy individuals (50  $\mu\text{l}$ ); 2) serum from patients suffering from ARDS (50  $\mu\text{l}$ ); or 3) BALF from ARDS patients (40  $\mu\text{l}$ ). In the set of additional experiments, 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  $\mu\text{M}$  final concentration) (25) or the selective C5aR antagonist AcF-[Opd-ChaWR (10  $\mu\text{M}$  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 TNF- $\alpha$  Ab (0.2  $\mu\text{g}/\mu\text{l}$  final concentration HUMIRA, Abbott Laboratories) or with IL-6 mouse-anti-human mAb (10  $\mu\text{g}/\text{ml}$  final concentration, MAB 206; R&D Systems). All the substances used in these studies were endotoxin free, as determined by a *Limulus* amoebocyte assay (Sigma-Aldrich), and the effects were found to be dose dependent, reaching peak activity at the doses chosen for this study. In addition, none of the reagents, agonists, or antagonists alone exhibited procoagulant activity.

### 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 spliced TF (asTF) (27), and to distinguish possible differences in TF among the various cell samples. In each sample, TF, asTF, and GAPDH mRNA sequence-specific primers and probes for detection were applied in conditions as previously described (15, 28).

The  $2^{-\text{DDC}_T}$  method (29) was used for quantification of the target genes (TF and asTF). In brief, we normalized the amount of target gene (TF or 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  $\text{DC}_T$  and  $\text{DDC}_T$  values, were used for the  $2^{-\text{DDC}_T}$  equation. Given the



**FIGURE 1.** TF-dependent procoagulant properties of BALF and the ability of BALF to induce procoagulant activity in neutrophils as demonstrated by mPT analyses. Average mPT values  $\pm$  SD of ARDS BALF supernatants (120  $\mu$ l, bar 3) and ARDS BALF supernatants intermixed with anti-TF mAb (bar 4). 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  $\pm$  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 ( $n = 7$ ).

limited sample number, a nonparametric Mann-Whitney  $U$  test (paired) was used to compare data within each group. The level of statistical significance was set to  $p < 0.05$ .

#### APAAP immunostaining

Immunocytochemical staining for TF was performed using the APAAP method, as previously described (30). An IgG1 mouse anti-human TF mAb (No. 4509; American Diagnostica) was used in this study. An IgG1 anti-CD19 mAb (M0740; DAKO) was used as a negative control.

#### FACS analysis of BALF cells and neutrophils isolated from peripheral blood

Approximately  $5 \times 10^5$  cells originating from BALF pellets and the same number of neutrophils isolated from peripheral blood were fixed and permeabilized for intracellular staining using the FIX & PERM system (Caltag Laboratories) according to the manufacturer's instructions. Cells were incubated with 1.8  $\mu$ g of the mouse anti-human TF mAb (4509) for 5 h at 4°C, then washed and incubated for 30 min at 4°C with FITC-labeled goat anti-mouse Ab (555988; BD Pharmingen). FITC-labeled mouse IgG1 (BD Biosciences) was used as an isotype control. Flow cytometry was performed using a FACScan with CellQuest software (BD Biosciences), and neutrophils were identified by forward- and side-scatter characteristics.

#### Western blot analysis

Cell lysates were prepared from  $\sim 2 \times 10^6$  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  $-20^\circ\text{C}$ . Western blot analysis was performed as previously described (15).

#### 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 (Panomics). 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 (GRO $\alpha$ ), IFN- $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40 and p70), IL-13, IL-17, IFN- $\gamma$  inducible protein of 10kD, leptin, MCP-3, MIP-1 $\alpha$ , MIP-1 $\beta$ , nerve growth factor, platelet derived growth factor BB, RANTES, TNF- $\alpha$ , TNF- $\beta$ , 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 (CGTLQKKIEEIAAKYKHSVVKK and CCV VASQLRANISHKDMQLGR, 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  $\pm$  SD, except for C5a and cytokine concentrations, which are presented as medians and range. Statistical analyses

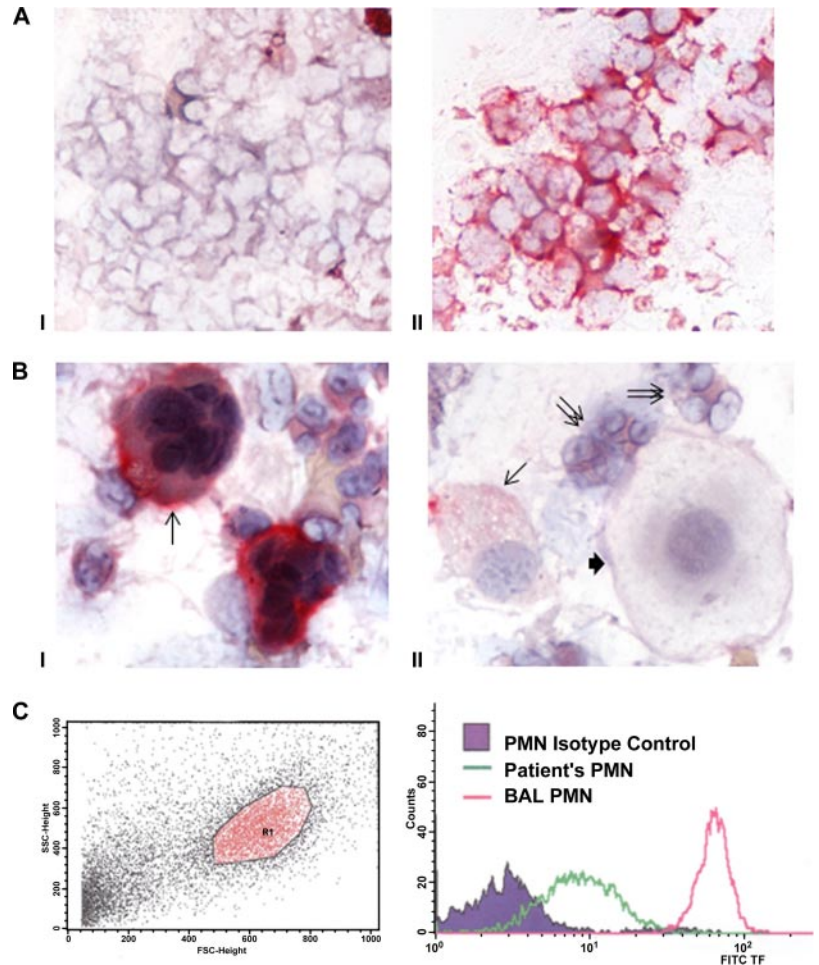
Table I. BALF cell characteristics

Sample ID	Total BALF Cells	Analyzed BALF Volume (ml)	Cell Populations <sup>c</sup>			
			PMN (%)	Lymphocytes (%)	Alveolar and Multi-Nucleated Phagocytes (%)	Eosinophils (%)
1	$4 \times 10^6$	18	76	6	14	4
2	$15 \times 10^6$	15 <sup>a</sup>	92 <sup>b</sup>	3	5	
3	$14 \times 10^6$	20 <sup>a</sup>	88 <sup>b</sup>	3	8	1
4	$8 \times 10^6$	20 <sup>a</sup>	80	5	13	2
5	$10 \times 10^6$	20 <sup>a</sup>	70	7	21	2
6	$9 \times 10^6$	25 <sup>a</sup>	81 <sup>b</sup>	4	15	
7	$11 \times 10^6$	25 <sup>a</sup>	84 <sup>b</sup>	4	9	3
Means			$81.57 \pm 7.34$	$4.57 \pm 1.51$	$12.14 \pm 5.30$	$1.71 \pm 1.50$

<sup>a</sup> Double gradient centrifugation was also performed to obtain pure PMN population for quantitative real-time PCR (purity:  $96.57 \pm 1.27$ ).

<sup>b</sup> FACS analysis was available in these BALF.

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



**FIGURE 2.** TF expression in BALF cells. *A*, TF immunostaining of peripheral blood neutrophils (I) and BALF neutrophils (neutrophil purity, 88%) (II) from an ARDS patient ( $n = 7$ ). *B*, Multinucleated cells (arrow) strongly positive for TF expression were observed mainly in the BALF of two patients (I). Alveolar macrophages with diffuse cytoplasmic staining (arrow) and large negative atypical pneumocyte type II (small arrow) are surrounded by positive neutrophils (double arrows) (II). *C*, Representative FACS analysis ( $n = 4$ ) of TF expression in neutrophils from peripheral blood (green histogram) and BALF fluid (red histogram). Purity of neutrophil populations obtained from BALF was 88%. Isotype negative control is shown as filled purple histogram.

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*

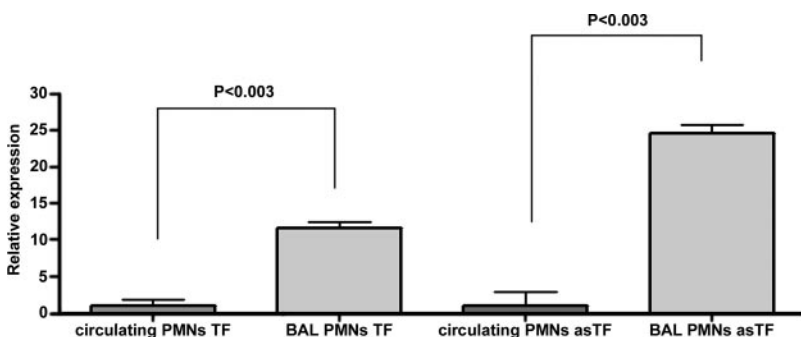
To evaluate the procoagulant activity of BALF from ARDS patients, we measured the mPTs of BALF supernatants. Preliminary experiments showed that the procoagulant activity of BALF was dose dependent, reaching maximal levels when 150  $\mu$ l of BALF was used for the assay; 40  $\mu$ l of BALF had no effect on the mPTs, when compared with control values (data not shown). For additional experiments, a dose of 120  $\mu$ l of BALF was used, because this amount of fluid induced significant shortening ( $p < 0.001$ ) of

the mPT ( $23.91 \pm 1.30$  s; Fig. 1, bar 3) when compared with the mPT of supernatants from neutrophils incubated with serum from healthy individuals ( $31.62 \pm 0.45$  s; Fig. 1, bar 2), or PBS alone ( $32.11 \pm 0.58$  s; Fig. 1, bar 1).

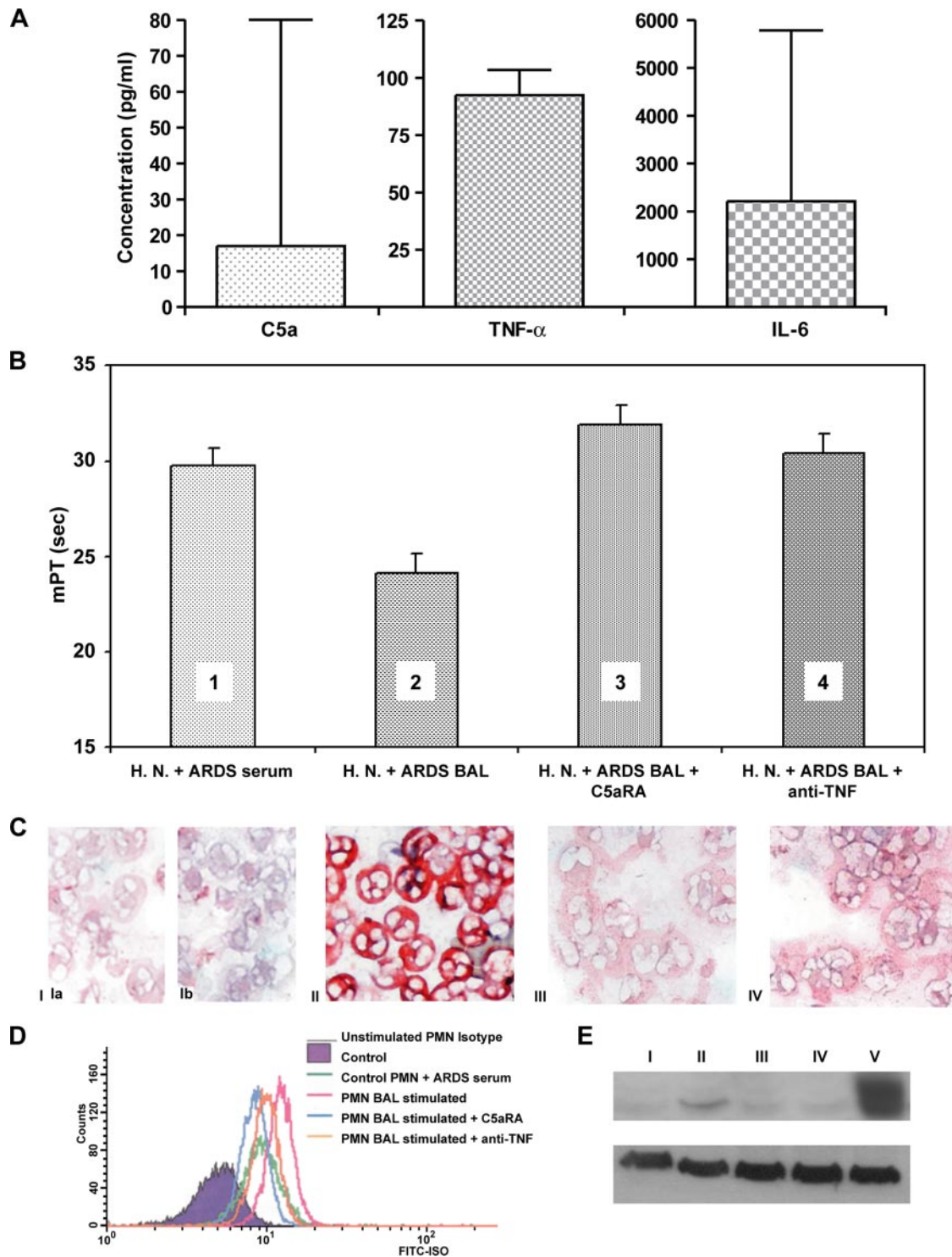
These assays further demonstrated that the procoagulant properties of BALF are dependent on the presence of functionally active TF, because preincubation of BALF supernatants with neutralizing anti-TF mAb completely abolished the procoagulant activity ( $31.95 \pm 0.74$  s; Fig. 1, bar 4).

*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



**FIGURE 3.** The  $2^{-DDC_T}$  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  $5.652 \pm 0.85$  vs  $8.798 \pm 0.94$  of TF blood PMNs and  $DC_T$  of asTF BALF-purified neutrophils  $11.435 \pm 1.10$  vs  $16.054 \pm 1.97$  of asTF blood PMN).



**FIGURE 4.** BALF C5a or TNF- $\alpha$  inhibition abolishes TF-dependent procoagulant activity. *A*, The median and range of concentrations for C5a, TNF- $\alpha$ , 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- $\alpha$  Ab (*bar 4*). Bars represent the average of mPT values  $\pm$  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- $\alpha$  Ab (*IV*). Images show immunostaining with the use of anti-TF Ab. Binding of anti-TF Ab was visualized by APAAP method (magnification  $\times 1000$ ). 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- $\alpha$  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  $\mu$ l BALF; *Lane III*: H.N. pretreated with C5aRA and stimulated with BALF. *Lane IV*: H.N. incubated with BALF and anti-TNF- $\alpha$ . *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  $96.57 \pm 1.27$ ; 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 asTF being the dominant isoform (Fig. 3).

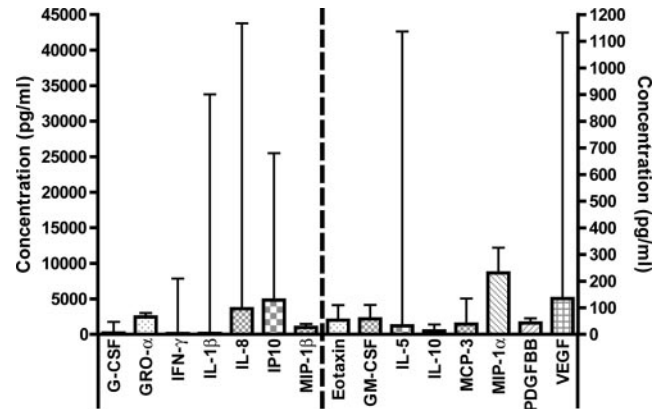
#### Mediators present in alveolar fluid in patients with ARDS induce the expression of TF in neutrophils

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  $\mu$ l 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  $24.13 \pm 1.31$  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 ( $31.73 \pm 0.37$  s; Fig. 1, bar 6).

Conversely, sera from ARDS patients did not induce significant procoagulant activity in neutrophils isolated from healthy individuals ( $29.69 \pm 1.64$  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- $\alpha$ 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- $\alpha$  (33) and IL-6 (33), accumulates in BALF of ARDS patients. Our studies confirmed that C5a, TNF- $\alpha$ , and IL-6 are indeed detectable in BALF from ARDS patients (Fig. 4A). Therefore,



**FIGURE 5.** Levels of cytokines in BALF. Medians and ranges of concentrations (pg/ml) for G-CSF, GRO $\alpha$ , IFN- $\gamma$ , IL-1 $\beta$ , IL-8, IFN- $\gamma$  inducible protein of 10kD, MIP-1 $\beta$ , eotaxin, GM-CSF, IL-5, IL-10, MCP-3, MIP-1 $\alpha$ , platelet derived growth factor BB, and vascular endothelial growth factor detectable in BALF from ARDS patients are shown ( $n = 7$ ). Due to high differences in the levels of various cytokines in ARDS BALF, two (right and left) panels separated by a dashed line are presented for better clarity. Values on the left y-axis refer to cytokines in the left panel, whereas values on the right y-axis refer to cytokines in the right panel.

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- $\alpha$ , or IL-6. The mPT analysis showed that the blockade of C5a or TNF- $\alpha$  signaling inhibited BALF-induced procoagulant activity of neutrophil supernatants and resulted in significant increase ( $p < 0.001$ ) of the mPT values to  $31.91 \pm 0.64$  s (Fig. 4B, bar 3) and  $30.43 \pm 0.52$  s (Fig. 4B, bar 4), respectively, when compared with the mPT of neutrophils from healthy volunteers incubated with BALF ( $24.13 \pm 1.31$  s; Fig. 4B, bar 2). Thus, the blockade of C5a or TNF- $\alpha$  signaling returned the mPT to values for control cells stimulated with ARDS serum ( $29.69 \pm 1.64$  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- $\alpha$  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, GRO $\alpha$ , MIP-1 $\alpha$ , MIP-1 $\beta$ , 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- $\alpha$  (Fig. 4), IL-1 $\beta$ , 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- $\alpha$ , 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 endotoxemia-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- $\alpha$ , 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- $\alpha$  suppression can decrease lung injury (45). However, the recruitment of neutrophils to the ARDS-affected lungs was reported to be TNF- $\alpha$  independent (45). Like C5a, TNF- $\alpha$  acts as a strong stimulator of TF expression in leukocytes and endothelial cells (9). Therefore, we also investigated whether TNF- $\alpha$ -mediated signaling contributes to the production of TF in pulmonary alveoli in ARDS patients.

A role for both TNF- $\alpha$  and C5a in ARDS-associated up-regulation of TF was further supported by our demonstration that pharmacological blockade of TNF- $\alpha$  and C5a signaling in neutrophils 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- $\alpha$  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- $\alpha$  both stimulate neutrophils directly or whether C5a acts only as an upstream regulator of TNF- $\alpha$ .

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- $\alpha$ -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- $\alpha$  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.

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## Disclosures

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