ExoU-induced procoagulant activity in *Pseudomonas aeruginosa*-infected airway cells

M.C. Plotkowski, L.F.P. Feliciano, G.B.S. Machado, L.G. Cunha Jr, C. Freitas, A.M. Saliba and M.C. de Assis

ABSTRACT: The present study addressed the question whether ExoU, a *Pseudomonas aeruginosa* toxin with phospholipase A2 (PLA2) activity, may induce airway epithelial cells to overexpress tissue factor (TF) and exhibit a procoagulant phenotype.

Cells from the human bronchial epithelial BEAS-2B line were infected with an ExoU-producing *P. aeruginosa* strain, pre-treated or not with the cytosolic PLA2 inhibitor methylarachidonyl fluorophosphate (MAFP), or with two ExoU-deficient mutants. Control noninfected and infected cells were assessed for the expression of: 1) TF mRNA by RT-PCR; 2) cell-associated TF by enzyme immunoassay and flow cytometry; 3) procoagulant activity by a colorimetric assay; and 4) microparticle-associated TF by flow cytometry. An enzyme immunoassay was also used to assess cell-associated TF in lung extracts from mice infected intratracheally with ExoU-producing and -deficient bacteria.

Cells infected with the wild-type bacteria had higher levels of TF mRNA, cell-associated TF expression, procoagulant activity and released microparticle-associated TF than cells infected with the mutants. Bacterial treatment with MAFP significantly reduced the expression of TF by infected cells. Lung samples from mice infected with the wild-type bacteria exhibited higher levels of cell-associated TF and procoagulant activity.

The present results demonstrate that ExoU may contribute to the pathogenesis of lung injury by inducing a tissue factor-dependent procoagulant activity in airway epithelial cells.

KEYWORDS: ExoU, lung injury, procoagulant activity, *Pseudomonas aeruginosa*, sepsis, tissue factor

Pseudomonas aeruginosa is one of the leading causes of Gram-negative nosocomial pneumonia. *P. aeruginosa* hospital-acquired pneumonia often results in sepsis, a severe clinical syndrome characterised by a generalised activation of inflammation and co-agulation pathways.

The lungs are among the most frequently affected organs during sepsis [1], and characteristically show fibrin deposition in alveolar and interstitial compartments [2]. Besides compromising the lung gas-exchange barrier, alveolar clotting processes are harmful because neutrophils and fibroblasts may be activated further by thrombin and fibrin degradation products, contributing to further tissue damage. Moreover, surfactant components may be incorporated into polymerising fibrin with subsequent loss of surface activity and alveolar instability [3]. However, the mechanisms that control fibrin deposition in patient airways remain poorly understood.

Tissue factor (TF; CD142), the major physiological initiator of the coagulation cascade, is an integral membrane protein expressed on a number of cells not in direct contact with blood. However, in both monocytes and endothelial cells, TF can be readily up-regulated by proinflammatory cytokines, bacterial lipopolysaccharide (LPS) and many other proinflammatory stimuli [4], giving these cells a prothrombotic phenotype. TF is fully functional when expressed on cell surfaces and binds factor VII, supporting its allosterical activation to factor VIIa. The enzymatic TF–VIIa complex then initiates the downstream clotting events that culminate in the conversion of prothrombin to thrombin. Thrombin proteolytically cleaves fibrinogen, yielding fibrin monomers that polymerise into a stable clot required for physiological haemostasis.

TF is also present in human tissues with large procoagulant activity, such as brain and placenta, as well as in multiple lung cells, including

bronchoalveolar macrophages and alveolar and bronchial epithelial cells [5, 6]. TF expression in alveolar cells has long been known to be modulated by inflammatory stimuli [4, 7], but the ability of the alveolar epithelium to initiate intra-alveolar coagulation and fibrin deposition through upregulation of active TF has only been addressed recently [8].

TF can also be released from different cell types, and circulates in extracellular fluids as a soluble fluid-phase protein [9] or associated with microparticles [10, 11]. Microparticles are vesicles shed from the blebbing plasma membrane of various cell types. In body fluids, they constitute reliable hallmarks of cell activation and/or damage. Microparticles exhibit anionic phospholipids, chiefly phosphatidylserine, at their surface, which provide a catalytic surface promoting the assembly of the enzyme complexes of the coagulation cascade. Hardly detectable in the peripheral blood of healthy individuals, procoagulant TF-bearing microparticles circulating at elevated levels are often associated with thrombotic propensity [11, 12]. In contrast with the well-documented thrombogenicity of microparticle-associated TF, the physiological activity of the soluble fluid-phase TF is quite unclear because TF requires association with anionic lipids to become procoagulant [13].

Prominent among the virulence factors involved in *P. aeruginosa* pathogenicity is ExoU, a toxin with phospholipase A2 (PLA2) activity that is injected directly into eukaryotic cells *via* the type III secretory system [14]. Previous studies from the current authors' group have highlighted the ability of ExoU to induce the release of large amounts of free arachidonic acid and eicosanoids, and to elicit a potent inflammatory response of airway cells [15, 16].

Studies carried out by KURAHASHI *et al.* [17] showed the critical role played by ExoU in the pathogenesis of sepsis occurring following *P. aeruginosa* pneumonia. However, the ability of ExoU to activate the coagulation cascade in animal airways has not yet been investigated.

The present study addressed the question of whether ExoU would upregulate the expression of TF by airway epithelial respiratory cells, thereby modulating their procoagulant activity. The current study was further motivated by studies reporting the modulation of TF-dependent procoagulant properties of human monocytes by arachidonic acid and both lipoxygenase- and cyclooxygenase-derived eicosanoids [18, 19].

MATERIAL AND METHODS

Bacterial strains and culture conditions
The laboratory *P. aeruginosa* PA103 strain and its ExoU-deficient PA103Δ*exoU* mutant [15] were used throughout the current study. An *exoU*-depleted mutant complemented with an *exoU* gene with a site-specific mutation in the PLA2 catalytic site (PA103 ΔUT/S142A), a generous donation of A. Hauser (Northwestern University, Chicago, IL, USA), was also used.

Bacteria were grown in Luria–Bertani broth at 37°C for 14–16 h under mild agitation, harvested by centrifugation and resuspended in M-199 cell culture medium (Sigma-Aldrich, St Louis, MO, USA) such that the suspension's absorbance at a wavelength of 640 nm was 0.1, corresponding to ~10^8 colony forming units (CFU)·mL^{-1}.

Cell culture and infection
Human bronchial epithelial cells from the BEAS-2B line were cultured in M-199 cell culture medium containing 10% foetal calf serum, glutamine and antibiotics (complete culture medium). Confluent cultures were trypsinised, cells were suspended in complete culture medium, seeded in 24-well (0.4×10^5 cells·well^{-1}) tissue culture plates and cultured for 48 h. Cells were then infected at a multiplicity of infection of ~100 bacteria·cell^{-1}. Since translocation of effector proteins from the type III secretory system depends on close contact between bacteria and host cells, bacteria were centrifuged ($1,000 \times g$ for 10 min) onto the cell monolayers prior to incubation at 37°C for 1 h. Cells were then immediately processed or incubated with culture medium containing gentamicin at 300 μg·mL^{-1} for different periods. In some assays, bacteria were treated for 30 min with 100 μM of the cytosolic PLA2 inhibitor methyl arachidonyl fluorophosphonate (MAFP), before addition to the cell cultures.

Detection of TF mRNA by RT-PCR
Total RNA was isolated from noninfected (control) or from BEAS-2B cells infected for 1 h using the QIAGEN RNeasy kit (QIAGEN, Hilden, Germany). cDNA was synthesised from total RNA by reverse transcription with the SuperScript^{TM} First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was subjected to the following PCR conditions: denaturation at 95°C for 2 min; and 30 (TF) or 25 (β-actin) cycles of denaturation at 95°C for 45 s, annealing at 50°C for 45 s and extension at 72°C for 45 s. An additional extension step of 5 min at 72°C was carried out after the last cycle. The primers used in the reactions were: 5'-CCC GAA CAG TTA ACC GGA AGA-3' (TF sense); 5'-GCT CCA ATG ATG TAG AAT ATT TCT CTG A-3' (TF antisense); 5'-CCT CGC CTT TGC CGA TCC-3' (β-actin sense) and 5'-GGA TCT TCA TGA GGT AGT CAG TC-3' (β-actin antisense). PCR products were subjected to electrophoresis in a 2% agarose gel and densitometry was performed using LabImage software (Kaplan GmbH, Halle, Germany).

Detection of cell-associated TF
The IMUBIND Tissue Factor ELISA kit (American Diagnostica, Stamford, CT, USA) was used to quantify TF in both extracts and supernatants from BEAS-2B cells infected for 1 h and incubated with the gentamicin-containing culture medium for an additional 2 h as well as in extracts from mice lungs at 24 h after intratracheal inoculation of *P. aeruginosa* suspensions, according to the manufacturer's instructions.

Detection of membrane-bound TF
Cell cultures infected for 1 h and incubated with the gentamicin-containing culture medium for additional 2 h were detached from the microplate wells with 0.05% EDTA, fixed with 4% paraformaldehyde in PBS, incubated with an anti-TF-fluorescein isothiocyanate (FITC) complex (American Diagnostica) and analysed with a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Functional relevance of cell-associated TF
TF exists in microdomains on the cell surface with a mixed population of active and encrypted proteins. Whereas TF can

be detected on the surface of unactivated cells, its full procoagulant activity is not. Therefore, two different approaches were used to investigate whether ExoU would induce airway cells to exhibit a procoagulant activity. In both, cells were infected for 1 h and incubated with the gentamicin-containing culture medium for an additional 2 h. In the first approach, noninfected and infected cells were dissociated from the microplate wells with 0.05% EDTA, rinsed, suspended in PBS and 10^3 cells were incubated with pooled citrated plasma obtained from human volunteers at 37°C for 2 min. Clot time was measured in duplicate as time necessary for clot to form (detected by the naked eye) following the addition of 25 mM $CaCl_2$.

In the second approach, control and infected cells were lysed by repeated freeze–thaws in a buffer containing 50 mM Tris-HCl, 100 mM NaCl, 0.1% Triton X-100, pH 7.4, and TF was extracted in the buffer for 18 h at 4°C under mild agitation. Procoagulant activity in cell lysates was then assessed with the Actichrome TF activity kit (American Diagnostica), according to the manufacturer's instructions.

Detection of TF-containing microparticles

Supernatants from controls and from cultures infected for 1 h and treated with the gentamicin culture medium for 2 h were centrifuged to remove cell debris. Supernatants were further centrifuged at $17,500 \times g$ for 30 min at 15°C, to obtain microparticles. Pellets were washed, treated simultaneouly with anti-TF-FITC and annexin V-Alexa Fluor 647 complexes (Invitrogen) for 30 min in ice and washed once. Microparticles were resuspended in PBS containing 1% bovine serum albumin and analysed for 1 min in a FACScalibur flow cytometer. The region corresponding to shed microparticles was gated in side scatter *versus* fluorescent intensity dot plot representations by using, as reference, a mix of fluorescent beads of diameters to cover the microparticles (0.5 and 0.9 µm) and platelets (3.0 µm; Megamix; Biocytex, Marseille, France), according to the manufacturer's instructions.

Detection of supernatant procoagulant activity

Procoagulant activity of cell culture supernatants obtained after centrifugation for 10 min to remove cell debris (microparticle-enriched supernatants) or after further centrifugation at $17,500 \times g$ for 30 min (microparticle-free supernatants) was assessed with a chromogenic assay (Actichrome TF activity kit).

In vivo *assays*

Female Swiss mice aged 8–12 weeks were anaesthetised with a mixture of ketamine (65 mg·kg^{-1}) and xylazine (13 mg·kg^{-1}) administered *i.p.*, and 10^4 CFU of PA103 or PA103Δ*exoU* in 50 µL LPS-free saline were instilled into their tracheas. At 24 h post-infection, mice were euthanised by *i.p.* injection of sodium pentobarbital. Their airways were then washed once with 1 mL of PBS. Lungs were next washed free of blood by perfusing the heart and lungs with 10 mL of cold PBS and kept at -70°C for ulterior treatment with lysis buffer, ELISA detection of TF and assessment of procoagulant activity, using the Actichrome TF activity kit, according to the manufacturer's instructions. All animal experiments were approved by the Animal Ethics Committee of the State University of Rio de Janeiro (Rio de Janeiro, Brazil).

Statistical analysis

Statistical analysis was performed using a one-way ANOVA with Bonferroni's test to determine significant statistical differences between groups, unless otherwise stated. p-values <0.05 were deemed to be significant.

RESULTS

ExoU enhanced the expression of TF by P. aeruginosa-infected cells

RT-PCR was performed to investigate whether *P. aeruginosa* infection and/or ExoU production could alter TF mRNA levels in airway epithelial cells. The data shown in fig. 1 show that TF transcripts were constitutively present in BEAS-2B cells and that infection for 1 h with the ExoU-producing wild-type bacteria increased the level of mRNA by ~45%, in comparison with noninfected and PA103Δ*exoU*-infected cells.
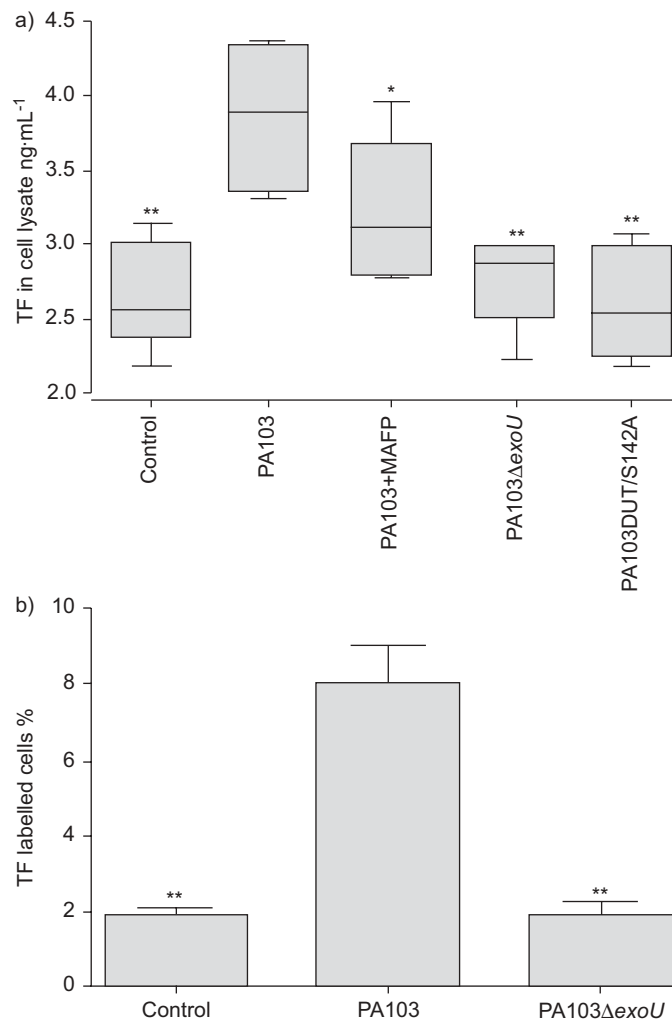
Infection with PA103 for 3 h increased the baseline expression of TF protein approximately 1.5-fold, as detected by ELISA assays in control culture lysates. Previous bacterial treatment with the PLA2 inhibitor MAFP reduced significantly the expression of TF by PA103-infected cells. Consistent with the
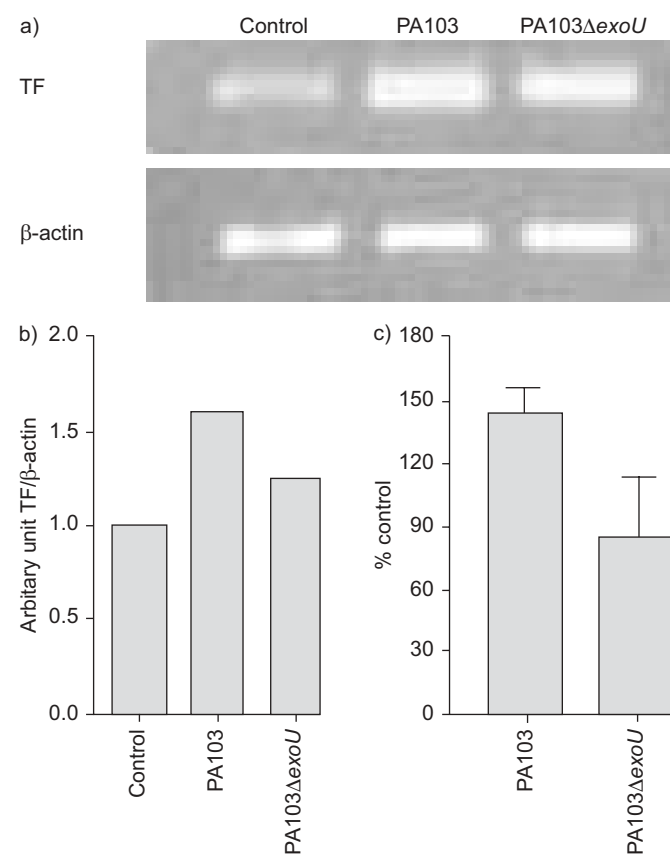
FIGURE 1. a) RT-PCR analysis of tissue factor (TF) and β-actin mRNA transcripts in control noninfected cells and in cells infected with the ExoU-producing PA103 bacterial strain and the ExoU-deficient mutant PA103Δ*exoU*, representative of four analyses carried out with similar results. b) Ratio of TF to β-actin internal control density in the agarose gel shown in (a). c) Increase of the relative TF/β-actin transcript densities in PA103 and PA103Δ*exoU*-infected cells in comparison with the relationship obtained in control cells. Data are presented as mean±SD of four different assays.

role of ExoU PLA2 activity in the modulation of TF expression, no increase in TF was detected in cells infected with bacteria deficient in *exoU* or with mutated PLA2 catalytic activity site (fig. 2a). The enhanced expression of TF in PA103-infected cells was further confirmed by fluorescence-activated cell sorting analysis of cells labelled with a monoclonal anti-TF antibody (fig. 2b).

The biological relevance of enhanced TF expression by PA103-infected cells was investigated next. As shown in figure 3a, clotting time was significantly lower and procoagulant activity was significantly higher in cells infected with ExoU-producing bacteria than in control cells or in cells infected with the *exoU* bacterial mutant (fig. 3a and b).

ExoU also enhanced the release of TF-bearing microparticles from infected cells

The potential of ExoU to modulate the release of TF from cell plasma membranes was assessed by ELISA. Figure 4a shows that the concentration of TF in supernatants from PA103-infected cells was about five-fold higher than in supernatants from control and PA103Δ*exoU*-infected cultures. Infection with the ExoU-producing bacteria also enhanced the release of microparticles from cell membranes (fig. 4b), and the percentage of microparticles expressing TF at their surfaces was significantly higher in supernatants from PA103-infected cells (fig. 4c). More importantly, a higher percentage of microparticles released after PA103 infection, besides exhibiting surface TF, was shown to bind annexin V (fig. 4d), a protein known for its interaction with negatively charged phospholipids, such as phosphatidylserine, one of the essential lipid cofactors for clotting. These TF-positive/annexin V-positive microparticles are likely to provide a catalytic surface promoting the assembly of the enzyme complexes of the coagulation cascade.

TF-bearing microparticles, but not soluble TF, accounted for the procoagulant activity of cell culture supernatants

Since conflicting results have been published relating to the contribution of the soluble isoform [9, 10, 14] and full-length TF
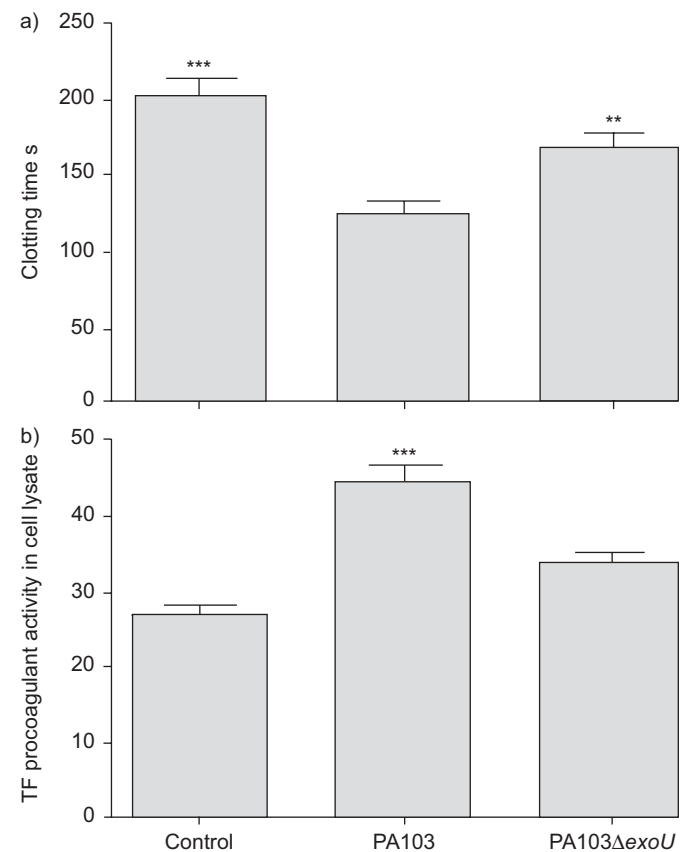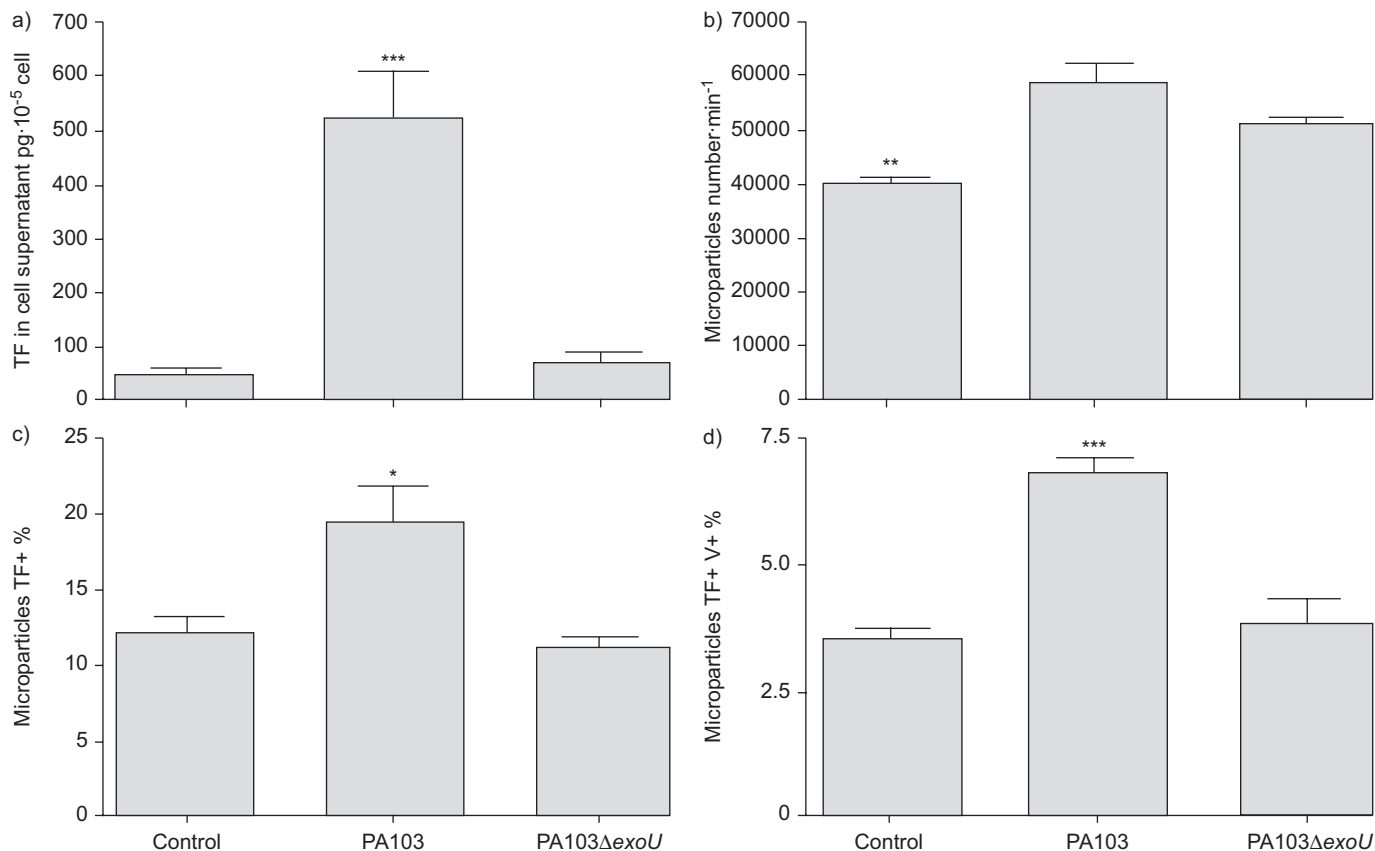
FIGURE 2. a) Concentration of tissue factor (TF) in lysates from control and *Pseudomonas aeruginosa*-infected airway epithelial cells. The boxes extend from the 25th–75th percentiles, with the horizontal line at the median value. Whiskers show the range of the data. b) Percentage of cells expressing surface TF, as determined by fluorescence-activated cell sorting analysis. Data are presented as mean±SD of the results from two different assays carried out in triplicate. *: p<0.05; **: p<0.01 when data were compared with the results obtained from PA103-infected cells.

FIGURE 3. Clotting time (a) and procoagulant activity (b) of control and *Pseudomonas aeruginosa*-infected airway epithelial cells. Data are presented as mean±SD of the results from three different assays carried out in tripicate. **: p<0.01; ***: p<0.001 when data were compared with the results obtained from PA103-infected cells.

FIGURE 4. a) Concentration of tissue factor (TF) in microparticle-enriched supernatants from control and *Pseudomonas aeruginosa*-infected airway epithelial cells. b) Number of microparticles in control and *P. aeruginosa*-infected cell culture supernatants submitted to fluorescence-activated cell sorting analysis for 1 min. c) Percentage of TF-positive microparticles; and d) percentage of TF positive/annexinV positive microparticles in supernatant of control and infected cells. Data are presented as mean±SD of the results from two assays carried out in triplicate. *: $p < 0.05$ and ***: $p < 0.001$ when data from control and PA103ΔexoU-infected cells were compared with the results from PA103-infected cultures (a, c, d). **: $p < 0.01$ when the results from control cultures were compared with those from cells infected with the wild-type bacteria (b).

associated with microparticles to thrombogenicity of extracellular fluids, the impact of each one on the procoagulability of airway epithelial cell supernatants was investigated. Figure 5 shows the results obtained with a chromogenic assay and shows that infection with the ExoU-producing bacteria led to an ~1.8-fold rise in TF activity in microparticle-enriched supernatants from PA103-infected cells, compared with non-infected cultures. When the effect of ExoU was assessed in microparticle-free culture supernatants, the thrombogenicity in supernatant from PA103-infected cultures dropped from 35.9±3.1 pM to 0.4±0.2 pM. No activity was detected in supernatants from controls and from cells infected with the ExoU-deficient mutant (data not shown). These results clearly show that TF-bearing microparticles, but not soluble TF, accounted for the thrombogenicity of cell culture supernatants.

Lung samples from mice infected intratracheally with ExoU-producing bacteria exhibited higher TF concentration and thrombogenicity

To ascertain the biological relevance of the *in vitro* results, the *in vivo* effect of ExoU on TF expression by lung cells was investigated. As shown in figure 6a, TF concentration in lung parenchyma from mice infected with the ExoU-producing PA103 strain was approximately three-fold higher than in

lungs from mice infected with the ExoU-deficient bacteria. Lysates of PA103-infected lung parenchyma also exhibited a higher procoagulant activity (fig. 6b), consistent with data obtained in *in vitro* assays.

DISCUSSION

The current study demonstrated the ability of ExoU to induce thrombogenic properties in airway epithelial cells through active transcriptional modulation of TF expression, and to enhance the release of procoagulant microparticles from infected cells. The present authors are unaware of any prior investigation that directly addressed this possibility. Since *in vitro* results were supported by similar finding with lysates of the lung parenchyma of PA103-infected mice, the current authors speculate that this ability may contribute to the clinical significance of this toxin, considered to be a marker for highly virulent *P. aeruginosa* isolates recovered from patients with hospital-acquired pneumonia [20] and bacteraemia [21].

Consistent with other studies, BEAS-2B airway cells were shown to express TF mRNA and protein constitutively. Such TF expression is likely to provide airway epithelial cells with the capability to generate a provisional fibrin matrix to favour their migration during the wound-healing process following bronchial epithelium injury, independently of plasma proteins [22].
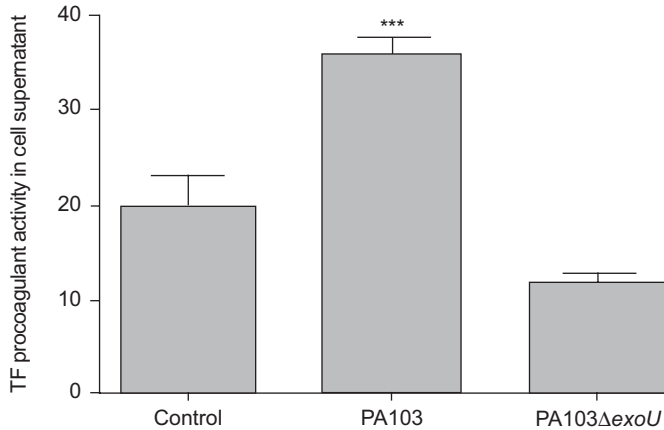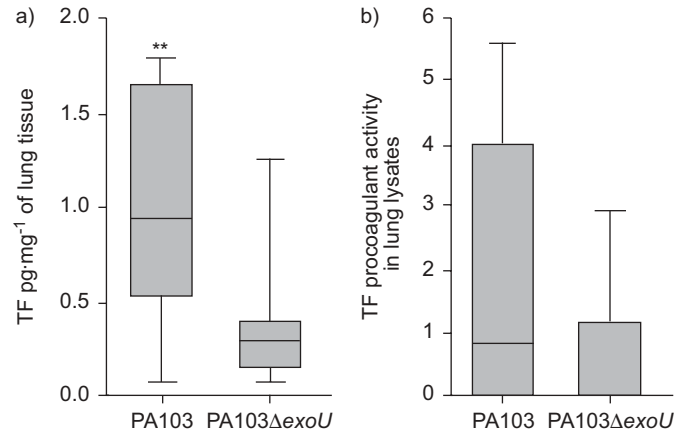
FIGURE 5. Procoagulant activity of microparticle-enriched supernatant of control and *Pseudomonas aeruginosa*-infected airway epithelial cells. Data are presented as mean $\pm$ SD of the results from two assays carried out in triplicate. ***: $p < 0.001$ when data were compared with the results from PA103-infected cultures.

FIGURE 6. a) Concentration of tissue factor (TF) and b) procoagulant activity detected in lung lysates from control and *Pseudomonas aeruginosa*-infected mice. The boxes extend from the 25th–75th percentiles, with the horizontal line at the median value. Whiskers show the range of the data obtained from animals infected with PA103 (n=10) or PA103Δ*exoU* (n=13). **: $p < 0.01$ when data were compared using the Mann–Whitney test.

Therefore, the initiation of clot formation by TF-bearing airway cells is believed to be essential for a rapid initial repair process. Ulterior leakage of plasma protein (including fibrinogen) into the airways would favour a potentially harmful propagation of the coagulation. Transitional fibrin itself may lead to deleterious effects by providing a matrix on which fibroblasts can also migrate and produce collagen, leading to accelerated pulmonary fibrosis. Additionally, fibrin can enhance local inflammatory response by increasing vascular permeability and activating endothelial cells to produce proinflammatory cytokines and other mediators. Thus, the local fibrin formation by TF-bearing airway cells may become a pathological event. Indeed, fibrin deposition in the lung parenchyma and airspaces is considered to be a hallmark of acute lung injury and acute respiratory distress syndrome [23].

The bronchoalveolar procoagulant response during severe infection has been shown to depend also on dysfunctional local anticoagulant pathways (*e.g.* antithrombin and the protein C system) and fibrinolytic activity, caused by high levels of plasminogen activator inhibitor-1 and reduced levels of plasminogen activator activity, as described by CHOI and co-workers [24, 25] in patients with ventilator-asociated pneumonia and experimental pneumonia in rats.

In the current report, cells infected with the ExoU-producing *P. aeruginosa* strain exhibited significantly higher TF content. Previous treatment of bacteria with MAFP, a PLA2 inhibitor, reduced their ability to upregulate TF production significantly, consistent with the phospholipase activity of ExoU. These *in vitro* findings were validated by the demonstration that both TF concentration and procoagulant activity in the lung parenchyma of PA103-infected mice were substantially higher than in the parenchyma of mice infected with the *exoU* mutant. Based on these results, it can be speculated that overexpression of TF elicited by bacterial infection can initiate local coagulation, contributing at least partially to the severe lung injury and increased mortality described in both patients [20] and experimental animals [17] with pneumonia caused by ExoU-producing *P. aeruginosa* strains.

Besides being expressed at plasma membranes, TF can also circulate associated with microparticles released from different cell types upon activation and/or damage. There is increasing evidence that microparticles carry proinflammatory cell membrane lipids and glycoproteins testifying to their cellular origin that can be transferred to neighbouring or remote cells bearing appropriate counter-receptors for ligands they harbour. The cellular effects depend on microparticle membrane and cytoplasmic composition and the nature of target cells, and can be as different as cell activation, enhancement of monocyte–endothelium adhesiveness, vascular dysfunction, induction of apoptosis and others [11, 12]. The implication of microparticles in inflammation is also well documented. For instance, microparticles are a source of substrates of secretory PLA2 for generation of lysophosphatidic acid, a potent proinflammatory mediator and platelet agonist. Interestingly, in sepsis, elevated levels of PLA2 activity in bronchoalveolar lavage fluids have been shown to have a prognostic value [26].

In the present study, ExoU was also shown to augment both the number and the proportion of TF- and posphatidylserine-bearing microparticles released by airway epithelial cells, and *in vitro* assays showed that these TF-bearing microparticles were procoagulant. It is conceivable that a similar *in vivo* augmentation of microparticle release would represent a second mechanism by which ExoU would increase the thrombotic propensity of *P. aeruginosa*-infected airway epithelial cells. However, knowing that microparticles are endowed with the potential to disseminate a wide range of different biological information, the pathophysiological role played by ExoU-induced microparticles may not be restricted to their procoagulant potential. For instance, microparticles have been shown both to be a rapid substrate for peroxidation and to carry biologically active oxidised phospholipids that elicit specific responses in endothelial cells [27]. Excitingly, the present authors have previously demonstrated the ability of ExoU to induce a significant peroxidation of cell membrane lipids from PA103-infected endothelial cells [28]. Increased

release of oxidised microparticles from *P. aeruginosa*-infected cells is likely to contribute to the progression of inflammatory processes elicited in the course of *P. aeruginosa* infection.

Different human pathogens [29, 30] have been shown to modulate the expression of tissue factor by monocytes and endothelial cells, thereby promoting vascular thrombosis but, to the current authors' knowledge, this is the first study reporting the bacterial up-regulation of tissue factor in airway epithelial cells. However, it is conceivable that ExoU may similarly enhance tissue factor expression by endothelial cells and that this ExoU ability during *Pseudomonas aeruginosa* bacteraemia could serve as a virulence factor promoting a hypercoagulable state frequently detected in sepsis. Studies to examine this hypothesis are currently in progress.

REFERENCES
1 Levi M, Keller TT, van Gorp E, Cate H. Infection and inflammation and the coagulation system. *Cardiovasc Res* 2003; 60: 26–39.

2 Martin GS, Bernard GR. Airway and lung in sepsis. *Intensive Care Med* 2001; 27: Suppl. 1, S63–S79.

3 Seeger W, Hübel J, Klapettek K, *et al.* Procoagulant activity in bronchoalveolar lavage of severely traumatized patients – relation to the development of acute respiratory distress. *Thromb Res* 1991; 61: 53–64.

4 Chu AJ. Tissue factor mediates inflammation. *Arch Biochem Biophys* 2005; 15: 123–132.

5 Welty-Wolf KE, Carraway MS, Ortel TL, Piantadosi CA. Coagulation and inflammation in acute lung injury. *Thromb Haemost* 2002; 88: 17–25.

6 Østerud B, Bjørklid E. Sources of tissue factor. *Semin Thromb Hemost* 2006; 32: 11–23.

7 Mackman N, Sawdey MS, Keeton MR, Loskutoff DJ. Murine tissue factor gene expression *in vivo*: tissue and cell specificity and regulation by LPS. *Am J Pathol* 1993; 143: 76–84.

8 Bastarache JA, Wang L, Geiser T, *et al.* The alveolar epithelium can initiate the extrinsic coagulation cascade through expression of tissue factor. *Thorax* 2007; 62: 608–616.

9 Bogdanov Y. Alternatively spliced human tissue factor: a circulating soluble thrombogenic protein. *Nat Med* 2003; 9: 458–462.

10 Giesen PL, Rauch U, Bohrmann B, *et al.* Blood-borne tissue factor: another view of thrombosis. *Proc Natl Acad Sci USA* 1999; 96: 2311–2315.

11 Morel O, Toti F, Hugel B, Freyssinet JM. Cellular microparticles: a disseminated storage pool of bioactive vascular effectors. *Curr Opin Hematol* 2004; 11: 156–164.

12 Hugel B, Martinez MC, Kunzelmann C, Freyssinet JM. Membrane microparticles: two sides of the coin. *Physiol* 2005; 20: 22–27.

13 Sturk-Maquelin KG, Niewland R, Romijn FP, Eijsman L, Hack CE, Sturk A. Pro- and non-coagulant forms of non-cell bound tissue factor *in vivo*. *J Thromb Haemost* 2003; 1: 1920–1926.

14 Sato H, Frank DW, Hillard CJ, *et al.* The mechanism of action of the *Pseudomonas aeruginosa*-encoded type III cytotoxin, ExoU. *EMBO J* 2003; 22: 2959–2969.

15 Saliba AM, Nascimento DO, Silva MC, *et al.* Eicosanoid-mediated proinflammatory activity of *Pseudomonas aeruginosa* ExoU. *Cell Microbiol* 2005; 7: 1811–1822.

16 Plotkowski MC, Brandão BA, de Assis MC, *et al.* Lipid body mobilization in the ExoU-induced release of inflammatory mediators by airway epithelial cells. *Microb Pathog* 2008; 45: 30–37.

17 Kurahashi K, Kajikawa O, Sawa T, *et al.* Pathogenesis of septic shock in *Pseudomonas aeruginosa* pneumonia. *J Clin Invest* 1999; 104: 743–750.

18 Eilertsen KE, Olsen JO, Osterud B. *Ex-vivo* regulation of endotoxin-induced tissue factor in whole blood by eicosanoids. *Blood Coagul Fibrinolysis* 2003; 14: 41–48.

19 Cadroy Y, Dupouy D, Boneu B. Arachidonic acid enhances the tissue factor expression of mononuclear cells by the cyclooxygenase 1 pathway. *J Immunol* 1998; 160: 6145–6150.

20 Schulert GS, Feltman H, Rabin SDP, *et al.* Secretion of the toxin ExoU is a marker for highly virulent *Pseudomonas aeruginosa* isolates obtained from patients with hospital-acquired pneumonia. *J Infect Dis* 2003; 188: 1695–1706.

21 Berthelot P, Attree I, Plesiat P, *et al.* Genotypic and phenotypic analysis of the type III secretion system in a cohort of *Pseudomonas aeruginosa* bacteremia isolates: evidence for a possible association between O serotypes and exo genes. *J Infect Dis* 2003; 188: 512–518.

22 Perrio MJ, Ewen D, Trevethick MA, Salmon GP, Shute JK. Fibrin formation by wounded bronchial epithelial ccell layers *in vitro* is essential for normal epithelial repair and independent of plasma proteins. *Clin Exp Allergy* 2007; 37: 1688–1700.

23 Wygrecka M, Jablonska E, Guenther A, Preissner KT, Markart P. Current view on alveolar coagulation and fibrinolysis in acute inflammatory and chronic interstitial lung diseases. *Thromb Haemost* 2008; 99: 494–501.

24 Choi G, Vlaar APJ, Schouten M, *et al.* Natural anticoagulants limit lipopolysaccharide-induced pulmonary coagulation but not inflammation. *Eur Respir J* 2007; 30: 423–428.

25 Choi G, Hofstra JJH, Roelofs JJTH, *et al.* Recombinant human activated protein C inhibits local and systemic activation of coagulation without influencing inflammation during *Pseudomonas aeruginosa* pneumonia in rats. *Crit Care Med* 2007; 35: 1362–1368.

26 Arbibr L, Vial D, Touqui L. Phospholipase A2 and acute respiratory distress syndrome. *In*: Uhl W, Nevalainem TJ, Büchler MW, eds. Phospholipase A2: basic and clinical aspects in inflammatory diseases. Prog Surg vol. 24. Basel, Karger, 1997; pp. 79–87.

27 Huber J, Vales A, Mitulovic G, *et al.* Oxidized membrane vesicles and blebs from apoptotic cells contain biologically active oxidized phospholipids that induce monocyte-endothelial interactions. *Arterioscler Thromb Vasc Biol* 2002; 22: 101–107.

28 Saliba AM, de Assis MC, Nishi R, *et al.* Implications of oxidative stress in the cytotoxicity of *Pseudomonas aeruginosa* ExoU. *Microbes Infect* 2006; 8: 450–499.

29 Bryant AE, Hayes-Schroer SM, Stevens DL. M type 1 and 3 group A streptococci stimulate tissue factor-mediated

procoagulant activity in human monocytes and endothelial cells. *Infect Immun* 2003; 71: 1903–1910.

30 Mattsson E, Heying R, van de Gevel JS, Hartung T, Beekhuizen H. Staphylococcal peptidoglycan initiates an inflammatory response and procoagulant activity in human vascular endothelial cells: a comparison with highly purified lipoteichoic acid and TSST-1. *FEMS Immunol Med Microbiol* 2008; 52: 110–117.