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REVISED VERSION

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

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Abstract

This report addressed the question whether ExoU, a *P. aeruginosa* toxin with PLA_2 activity, may induce airway epithelial cells to overexpress tissue factor (TF) and exhibit a procoagulant phenotype.

Cells from the BEAS-2B line were infected with an ExoU-producing *P. aeruginosa* strain, pretreated or not with the $cPLA_2$ inhibitor MAFP, or with two ExoU deficient mutants. Control noninfected and infected cells were assessed for the expression of i) TF mRNA by RT-PCR; ii) cell-associated TF by enzyme immunoassay and flow cytometry; iii) procoagulant activity by a colorimetric assay and iv) microparticle-associated TF by flow cytometry. 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 reduced significantly 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.

Our results demonstrate that ExoU may contribute to pathogenesis of lung injury by inducing a TF-dependent procoagulant activity in airway epithelial cells.

Key words: ExoU; Lung injury; procoagulant activity; *Pseudomonas aeruginosa*; sepsis; tissue factor

Introduction

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 characterized by a generalized activation of inflammation and coagulation 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 further activated by thrombin and fibrin degradation products, contributing to further tissue damage. Moreover, surfactant components may be incorporated into polymerizing 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 LPS and many other proinflammatory stimuli [reviewed in 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 VIIa. The enzymatic TF-VIIa complex then initiates the downstream clotting events which culminate in the conversion of prothrombin to thrombin. Thrombin proteolytically cleaves fibrinogen, yielding fibrin monomers that polymerize into a stable clot required for physiological hemostasis.

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, 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 recently addressed [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 physiologic

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 A_2 (PLA_2) activity that is injected directly into eukaryotic cells via the type III secretory system [14]. Previous studies from our group have highlighted the ability of ExoU to induce the release of high 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, in the animal airways, the coagulation cascade has not yet been investigated.

In the present study we addressed the question wether ExoU would upregulate the expression of TF by airway epithelial respiratory cells thereby modulating their procoagulant activity. Our 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 this study. We also used an *exoU* depleted mutant complemented with *exoU* gene with site-specific mutation in PLA_2 catalytic site (PA103 ΔUT/S142A), a generous donation of Dr. Alan Hauser (Northwestern University, Chicago).

Bacteria were grown in Luria-Bertani (LB) broth at 37°C for 14-16 h under mild agitation, harvested by centrifugation and resuspended in M-199 cell culture medium (Sigma-Aldrich) to $A_{640\ nm} = 0.1$, corresponding to about 10^8 CFU/mL.

Cell culture and infection

Human bronchial epithelial cells from the BEAS-2B line were cultured in M-199 cell culture medium containing 10% fetal calf serum, glutamine and antibiotics (complete culture medium). Confluent cultures were trypsinized, cells were suspended in complete culture medium, seeded in 24-well (0.4×10^5 cells per well) tissue culture plates and cultured for 48h. Cells were then infected at a multiplicity of infection of about 100 bacteria per cell. Since translocation of effector proteins from the type III secretory system depends on a close contact between bacteria and the host cells, bacteria were centrifuged (1,000 x g for 10 min) onto the cell monolayers prior to incubation at 37° C for 1h. Cells were then immediately processed or incubated with culture medium containing gentamicin at 300 μg/mL for different periods. In

some assays, bacteria were treated for 30 min with 100μM of the $cPLA_2$ inhibitor MAFP (methyl arachidonyl fluorophosphonate), before addition to the cell cultures.

Detection of TF mRNA by RT-PCR

Total RNA was isolated from non-infected (control) or from BEAS-2B cells infected for 1 h using the Qiagen Rneasy kit. cDNA was synthesized from total RNA by reverse transcription with the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) 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 (ß-actin) cycles of denaturation at 95˚C for 45 sec, annealing at 50˚C for 45 sec, and extension at 72˚C for 45 sec. 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' (ß-actin sense) and 5'-GGA TCT TCA TGA GGT AGT CAGTC-3' (ß-actin antisense). PCR products were subjected to electrophoresis in a 2% agarose gel and densitometry was performed using the LabImage software (Kaplan GmbH, Germany).

Detection of cell-associated TF

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

Detection of membrane-bound TF

Cell cultures infected for 1h 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-FITC complex (American Diagnostica) and analyzed with a FACScalibur flow cytometer (Becton Dickinson).

Functionnal relevance of cell-associated TF

TF exists in microdomains on the cell surface with a mixed population of active and encrypted protein. 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 1h and incubated with the gentamicin-containing culture medium for additional 2h. In the first approach, noninfected and infected cells were dissociated from the microplate wells with 0.05% EDTA, rinsed, suspended in PBS and $1x10^3$ cells were incubated with pooled citrated plasma obtained from human volunteers at 37^oC for 2 min. Clot time was measured in

duplicate as time necessary for clot to form, detected by naked eye, following the addition of 25mM Ca_2Cl.

In the second approach, control and infected cells were lysed by repeated freeze-thaws in a buffer containing 50mM 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 Inc.), according to the manufacturer´s instructions.

Detection of TF-containing microparticles

Supernatants from controls and from cultures infected for 1h and treated with the gentamicin culture medium for 2h were centrifuged to remove cell debris. Supernatants were further centrifuged at 17,500 x g for 30 min at 15°C, to obtain microparticles. Pellets were washed, treated simultaneouly with the 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% BSA (PBS-BSA) and analysed for 1 min in a FACScalibur flow cytometer. The region corresponding to shed microparticles was gated in side scatter vs. 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, Fr), 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 x g for 30 min (microparticle-free supernatants) was assessed with a chromogenic assay (Actichrome TF activity kit).

***In vivo* assays**

Female Swiss mice, 8–12 wk old were anesthetized with a mixture of ketamine (65 mg/kg) and xylazine (13 mg/kg) administered intraperitoneally and 10^4 CFU of PA103 or PA103Δ*exoU* in 50 μL of LPS-free saline were instilled into their tracheas. At 24h post infection, mice were euthanized by intraperitoneal 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 treatement with lysis buffer, ELISA detection of TF and assessement 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 (Brazil).

Statistical analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA) with the 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. Data exhibited in Fig. 1 show that TF transcripts were constitutively present in BEAS-2B and that infection for 1h with the ExoU-producing wild type bacteria increased in about 45% the level of mRNA, in comparison with non-infected and PA103Δ*exoU*-infected cells.

Infection with PA103 for 3h increased the baseline expression of TF protein in about 1.5-fold, as detected by ELISA assays in control culture lysates. Previous bacterial treatment with the PLA_2 inhibitor MAFP reduced significantly the expression of TF by PA103-infected cells. Consistent with the role of ExoU PLA2 activity in the modulation of TF expression, no increase in TF was detected in cells infected with the bacteria deficient in *exoU* or with mutated PLA_2 catalytic activity site (Fig. 2A). The enhanced expression of TF in PA103-infected cells was further confirmed by FACS analysis of cells labelled with a monoclonal anti-TF antibody (Fig. 2B).

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

ExoU enhanced also 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 assays. Data exhibited in Fig. 4A show that the concentration of TF in supernatants from PA103-infected cells was about 5-fold higher than in supernatants from control and PA103Δ*exoU*-infected cultures. Infection with the ExoU-producing bacteria enhanced also 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 microparticle, but not soluble TF, accounted for the procoagulant activity of cell culture supernatants

Since conflicting results have been published related to the contribution of the soluble isoform [9, 10, 14] and full-length TF associated with microparticles to thrombogenicity of extracellular fluids, we next investigated the impact of each one on the procoagulability of airway epithelial cell supernatants. Fig. 5 exhibits the results obtained with a chromogenic assay and shows that infection with the ExoU-producing bacteria led to about 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 $\pm$ 3.1 pM U to 0.4 $\pm$ 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 trombogenicity 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 our *in vitro* results, we looked for the *in vivo* effect of ExoU on TF expression by lung cells. As shown in Fig. 6A, TF concentration in lung parenchyma from mice infected with the ExoU-producing PA103 strain was about 3-fold higher than in lungs from mice infected with the ExoU-deficient bacteria. Lysates of PA103-infected lung parenchyma exhibited also a higher procoagulant activity (Fig. 6B), consistent with data obtained in our *in vitro* assays.

Discussion

In the current study we 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. We are unaware of any prior investigation that directly addressed this possibility. Since *in vitro* results were supported by similar finding with lysates of lung parenchyma of PA103-infected mice, we speculate 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 bacteremia [21].

Consistent with other studies, BEAS-2B airway cells were shown to constitutively express TF mRNA and protein. Such TF expression is likely to provide airway epithelial cells with the capability to generate a provisional fibrin matrix to favor their migration during the wound healing process following bronchial epithelium injury, independently of plasma proteins [22]. 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 favor a potentially harmful propagation of the coagulation.

Transitional fibrin itself may lead to deleterious effects by providing a matrix on which fibroblasts also can 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 the 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 (PAI)-1 and reduced levels of plasminogen activator activity, as described by Choi *et al.* [24, 25] in patients with ventilator-asociated pneumonia and experimental pneumonia in rats.

In this report, cells infected with the ExoU-producing *P. aeruginosa* strain exhibited significantly higher content of TF. Previous treatment of bacteria with MAFP, a PLA_2 inhibitor, reduced significantly their ability to up-regulate the TF production, consistent with the phospholipase activity of ExoU. These *in vitro* findings were validated by our demonstration that both TF concentration and procoagulant activity in lung parenchyma of PA103- infected mice were substantially higher than in parenchyma of mice infected with the *exoU* mutant. Based in these results, we speculate that overexpression of TF elicited by bacterial infection can initiate local coagulation contributing, at least partially, to 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 expressed at plasma membranes, TF can also circulate associated with microparticle released from different cell types upon activation and or damage. There are increasing evidences that microparticles carry pro-inflammatory cell membrane lipids and glycoproteins testifying to their cellular origin that can be transfered to neighboring or remote cells bearing appropriate counterreceptors for ligands they harbor. 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 implications of microparticles in inflammation is also well documented. For instance, microparticles are a source of substrates of secretory PLA_2 for generation of lysophosphatidic acid, a potent proinflammatory mediator and platelet agonist. Interestingly, in sepse, elevated levels of PLA_2 activity in bronchoalveolar lavage fluids have been shown to have a prognostic value [26].

In our 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 microparticles 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 many 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 rapidly substrate for peroxidation and to carry biologically active oxidized phospholipids that elicit specific response in endothelial cells [27]. Excintingly, we have previously shown the ability of ExoU to induce a significant peroxidation of cell membrane lipids from PA103-infected endothelial cells [28]. Increased release of oxidized microparticles from *P. aeruginosa*-infected cells is likely to contribute to the progression of inflammatory processes elicited in the course of *P. aeruginosa* infections.

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

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Figure legends

1. (A) RT-PCR analysis of TF and β-actin mRNA transcripts in control non-infected cells and in cells infected with the ExoU-producing PA103 and the ExoU-deficient mutant PA103Δ*exoU,* representative of four analysis carried out with similar results. (B). Ratio of TF to the β-actin internal control density in the agarose gel shown in A; (C) Increase of the relationship TF/β-actin transcript densities in PA103 and PA103Δ*exoU*-infected cells in comparison with the relationship obtained in control cells. Data are means ± SD of the results obtained in four different assays.

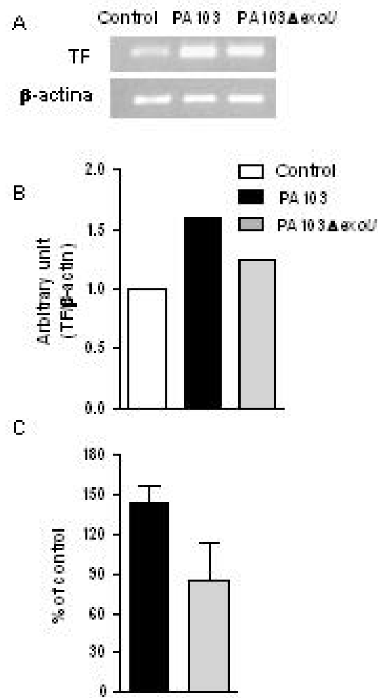

Fig. 1

2. (A) Concentration of TF in lysates from control and *P. aeruginosa*-infected airway epithelial cells. The boxes extend from the 25th percentile to the 75th percentile, 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 FACS analysis. Data are means ± SD of the results obtained in two different assays carried out in tripicate. *, p<0.05 and **, p<0.01 when data were compared with the results obtained with PA103-infected cells.

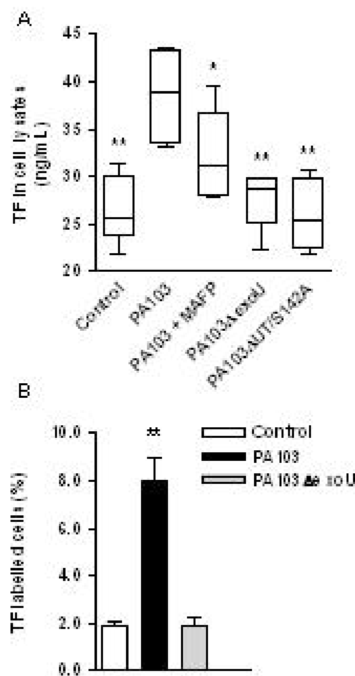

Fig. 2

3. Clotting time (A) and procoagulant activity (B) of control and *P. aeruginosa*-infected airway epithelial cells. Data are means ± SD of the results obtained in three different assays carried out in tripicate. **, p<0.01 and ***, p<0.001 when data were compared with the results obtained with PA103-infected cells.

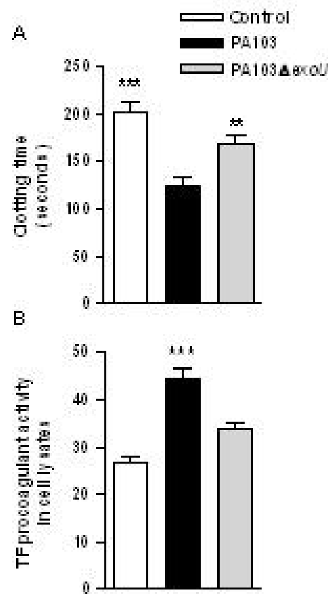

Fig. 3

4. (A) Concentration of TF in microparticle-enriched supernatants from control and *P. aeruginosa*-infected airway epithelial cells. (B) Number of microparticles in control and *P. aeruginosa*-infected cell culture supernatants submitted to FACS analysis for 1 min. (C) Percentage of TF positive microparticles and (D) Percentage of TF positive/annexinV positive microparticles in supernatant of control an infected cells. Data are means ± SD of the results obtained in two assays carried out in triplicate. *, p< 0.05 and ***, p<0.001 when data from control and PA103DexoU-infected cells were compared with the results from

PA103-infected cultures (A, C and D). **, p<0.01 when the results from control cultures were compared with those from cells infected with the wild type bacteria (B).

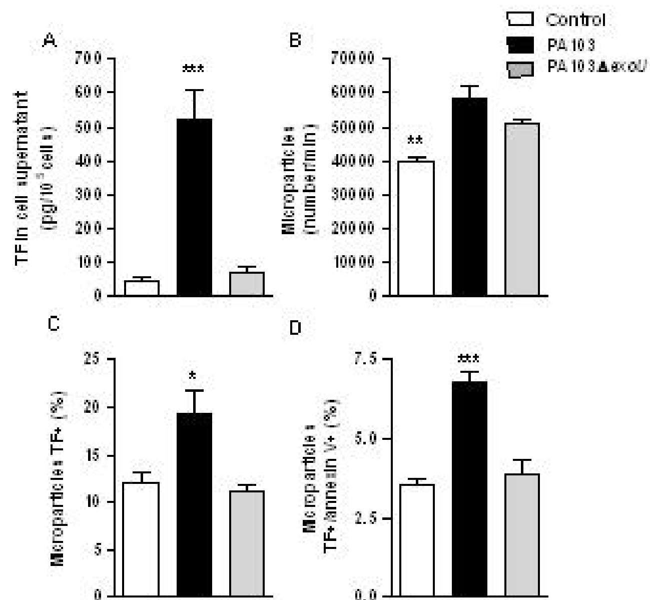

Fig. 4

5. Procoagulant activity of microparticle-enriched supernatant of control and *P. aeruginosa*-infected airway epithelial cells. Data are means ± SD of the results obtained in two assays carried out in triplicate. ***, p<0.001 when data were compared with the results from PA103-infected cultures.

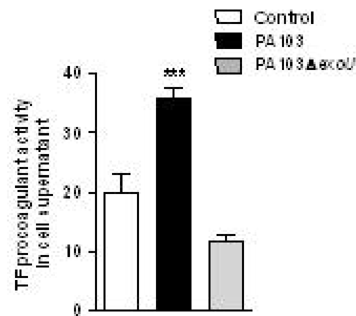

Fig.5

6. (A) Concentration of TF and (B) procoagulant activity detected in lung lysates from control and *P. aeruginosa*-infected mice. The boxes extend from the 25th percentile to the 75th percentile, wih 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 with each other with the Mann-Whitney test.

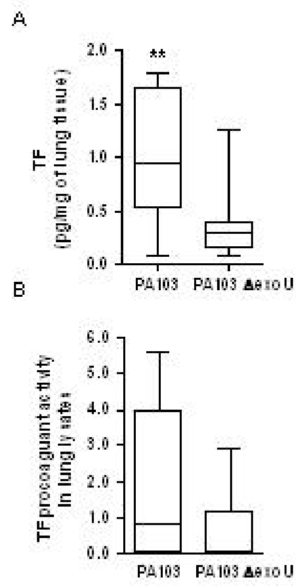

Fig. 6