Lung protective mechanical ventilation
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Chapter 14

The Role of Tissue Factor in Ventilator–Induced Pulmonary Coagulopathy in Mice


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Abstract

**Background:** Strategies blocking tissue factor (TF)–driven systemic coagulopathy improve outcome in sepsis models, at least in part via reducing systemic inflammation. Pharmacologically targeting pulmonary coagulopathy could be of benefit to patients with acute lung injury. We investigated whether TF plays a role in ventilator–induced lung injury (VILI).

**Methods:** Heterozygous TF knockout (TF+/−) mice and their wild–type (WT) littermates were tracheotomized and mechanically ventilated for 5 hours with either low tidal volumes (LV; ~7.5 ml/kg) or high tidal volumes (HV; ~15 ml/kg). Spontaneously breathing mice served as controls. Endpoints of VILI were pulmonary coagulopathy (thrombin–antithrombin complex levels), lung wet to dry (W/D) ratios, total protein level in bronchoalveolar lavage fluid (BALF), histopathology, neutrophil influx and pulmonary and plasma levels of cytokines/chemokines.

**Results:** Mechanical ventilation resulted in pulmonary coagulopathy in WT animals. TATc–levels in BALF were found to be lower in LV–animals as compared to HV–animals. The pro–coagulant response was largely attenuated in TF+/− mice in both ventilation groups (p < .05 versus WT animals). There were no differences in W/D ratio, BALF total protein levels, neutrophil influx and histopathology score between TF+/− and WT mice. Of note, BALF–levels of cytokines/chemokines were significantly higher in TF+/− as compared to WT mice. Systemic levels of cytokines/chemokines were not different between TF+/− mice and WT mice.

**Conclusions:** Coagulopathy is a feature of VILI, and TF seems to play an important role in the pathogenesis of disturbed alveolar fibrin turnover. However, relative TF deficiency does not reduce VILI; in respect to some endpoints of VILI, relative TF deficiency even seems to induce more inflammation.
**Introduction**

Mechanical ventilation is an indispensable tool in the treatment of patients with acute respiratory failure and is mandatory during general anesthesia. However, mechanical ventilation can induce lung injury in healthy lungs or aggravate pre-existing lung injury [1-3]. This phenomenon is frequently referred to as ventilator–induced lung injury (VILI) in animals or ventilator–associated lung injury (VALI) in human subjects [4]. All mechanically ventilated patients may be at risk for VALI.

Pulmonary coagulopathy is a characteristic finding in acute lung injury [5] and pneumonia [6], and is the result of localized tissue factor–mediated thrombin generation, impaired activity of natural coagulation inhibitors and depression of bronchoalveolar urokinase plasminogen activator–mediated fibrinolysis, caused by an increase of plasminogen activator inhibitors [7-9]. Pulmonary fibrin turnover is also influenced by mechanical ventilation [10,11]. Indeed, in a rat model of artificial pulmonary coagulopathy mechanical ventilation with high tidal volumes (VT) attenuates the fibrinolytic activity via an increase of plasminogen activator inhibitors [10]. Similar results are obtained in a model of endotoxin–induced lung injury [11]. In addition, mechanical ventilation with settings that have been shown to be harmful (high VT and no positive end–expiratory pressure [PEEP]) causes activation of bronchoalveolar coagulation, as reflected in a marked increase in thrombin–antithrombin complexes (TATc), soluble tissue factor (TF), and factor VIIa [12]. Lung–protective mechanical ventilation settings (low VT and PEEP) largely attenuate these changes in procoagulant activity within the airways.

The aim of this investigation was to determine whether TF plays a role in the pathogenesis of pulmonary coagulopathy in a VILI–model in mice. In addition, we determined whether attenuation of pulmonary fibrin production alters the local inflammatory response.

**Materials and Methods**

The study was approved by the Animal Care And Use Committee of the Academic Medical Center of the University of Amsterdam, Amsterdam, the Netherlands. Animal procedures were carried out in compliance with Institutional Standards for Human Care and Use of Laboratory Animals.

**Mice**

Experiments were performed in heterozygous TF knockout mice (TF+/-; n = 36) and their wild–type (WT; n = 36) littermates, aged 13 – 17 weeks, with weights ranging from 22 – 32 grams. These TF+/- mice, on a C57Bl/6 background [13], were bred and maintained at the animal care facility of the Academic Medical Center. Non–ventilated mice served as controls (n = 12 for either WT or TF+/- mice). Intubated and mechanically ventilated mice
were ventilated with 2 different mechanical ventilation strategies (see below). In total this results in 6 groups of animals (see below).

Instrumentation and anesthesia

Throughout the experiments rectal temperature was monitored and maintained between 36.0 – 37.5°C using a warming path. Anesthesia was achieved with intraperitoneal injection of a mix of ketamine (Eurovet Animal Health B.V., Bladel, the Netherlands), medetomidine (Pfizer Animal Health B.V., Capelle a/d IJssel, the Netherlands), and atropine (Pharmachemie, Haarlem, the Netherlands) (KMA). Induction anesthesia consisted of injection of KMA “induction”–mix: 7.5 µl per gram of body weight of 1.26 ml 100 mg/ml ketamine, 0.2 ml 1 mg/ml medetomidine, and 1 ml 0.5 mg/ml atropine in 5 ml normal saline. Maintenance anesthesia consisted of injection of 10 µl per gram body weight of KMA “maintenance”–mix, consisting of 0.72 ml 100 mg/ml ketamine, 0.08 ml 1 mg/ml medetomidine, and 0.3 ml 0.5 mg/ml atropine, in 20 ml normal saline. Maintenance–mix was administered via an intraperitoneal catheter (PE 10 tubing, BD, Breda, the Netherlands) every hour.

Mechanical ventilation strategies

A Y–tube connector, 1.0 mm outer diameter and 0.6 mm inner diameter (VBM Medizintechnik GmbH, Sulz am Neckar, Germany) was surgically inserted into the trachea under general anesthesia. Mice were placed in a supine position and connected to a human ventilator (Servo 900 C, Siemens, Solna, Sweden). Mice were pressure controlled ventilated with either an inspiratory pressure of 10 cmH₂O (resulting in lung–protective V₉ ~ 7.5 ml/kg; low V₉, LV₉) or an inspiratory pressure of 18 cmH₂O (resulting in injurious V₉ ~ 15 ml/kg; high V₉, HV₉). Respiratory rate was set at 110 breaths/min and 50 breaths/min with LV₉ and HV₉, respectively. These respiratory settings resulted in normal PaCO₂–values after 5 h of MV. PEEP was set at 2 cmH₂O with both mechanical ventilation strategies. The fraction of inspired oxygen was kept at 0.5. The inspiration to expiration ratio was kept at 1:1 throughout the experiment. A sigh (sustained inflation with 30 cmH₂O) for 5 breaths was performed every 30 minutes. Mice received an intraperitoneal bolus of 1 ml normal saline 1 hour before start of anesthesia and initiation of MV, followed by 0.2 ml sodium bicarbonate (200 mmol/l NaHCO₃) administered via the intraperitoneal catheter every 30 minutes until the end of MV.

Hemodynamic and ventilatory monitoring

Systolic blood pressure and heart rate were non–invasively monitored using a murine tail–cuff system (ADInstruments, Spenbach, Germany). Blood pressure and pulse were measured directly after start of MV, after 2.5 and 5 hour of MV. The data were recorded
on a data acquisition system (PowerLab/4SP, ADInstruments). Systolic blood pressure and heart rate were averaged from three consecutive measurements.

$V_T$ was checked hourly with a specially designed mice “Fleisch–tube” connected to the warmed plethysmograph system. The flow signal was integrated from a differential pressure transducer and data were recorded and digitized on–line using a 16–channel data–acquisition program (ATCODAS, Dataq Instruments Inc, ‘s Hertogenbosch, the Netherlands) and stored on a computer for post acquisition off line analysis. A minimum of 5 consecutive breaths was selected for analysis of the digitized $V_T$–signals.

**Study groups**

Non–ventilated control mice (WT and TF+/- mice) received half the dose of induction anesthesia, were spontaneously breathing and sacrificed after 5 hours.

LV$_T$–mice and HV$_T$–mice (WT and TF+/- mice) were mechanically ventilated for 5 hours and then sacrificed. Half of these mice were sacrificed and blood was drawn from the vena cava inferior into a sterile syringe, transferred to EDTA–coated tubes and immediately placed on ice. Subsequently, bronchoalveolar lavage fluid (BALF) was obtained from the right lung; the left lung was used to measure wet to dry ratios. In the other half of these mice blood was sampled from the carotid artery and used for blood gas analysis. The lungs of these mice were used for homogenate (right lung) and histopathology (left lung).

**Blood gas analysis**

For blood gas analysis, blood was immediately analyzed in a Rapidlab 865 blood gas analyzer (Bayer, Mijdrecht, the Netherlands). The other blood samples were centrifuged at 3000 rpm at 4°C for 10 min and the supernatants were aliquoted and frozen at -20°C until assayed.

**Lung wet to dry ratios**

For wet to dry (W/D) ratios the left lung was weighed and subsequently dried for three days in an oven at 65°C. The ratio of wet weight to dry weight represents tissue edema.

**Lung homogenates**

During sacrificing the right lung was removed and snap frozen in liquid nitrogen. These frozen specimens were suspended in 4 volumes of sterile isotonic saline and subsequently lysed in 1 volume of lysis buffer (150 mM NaCl, 15 mM Tris [tris(hydroxymethyl)aminomethane], 1 mM MgCl$_2$, 1mM CaCl$_2$, 1% Triton X-100, 100 μg/ml pepstatin A, leupeptin and aprotinin, pH 7.4) and incubated at 4°C for 30 min. Homogenates were spun at 3400 rpm at 4°C for 15 minutes after which the supernatants were stored at –20°C until assayed.
Bronchoalveolar lavage

Bronchoalveolar lavage fluid (BALF) was obtained by instilling three times 0.5 ml aliquots of saline by a 22-gauge Abbocath–T catheter (Abbott, Sligo, Ireland) into the trachea. Approximately, 1.0 ml of lavage fluid was retrieved per mouse and cell counts were determined using a hemacytometer (Beckman Coulter, Fullerton, CA). Subsequently, differential counts were done on citospin preparations stained with a modified Giemsa stain, Diff–Quick (Dade Behring AG, Düdingen, Switzerland). Supernatant was stored at -80°C for total protein level and thrombin–antithrombin complexes.

Histopathology

For histopathology lungs were fixed in 4% formalin and embedded in paraffin. 4μm sections were stained with hematoxylin–eosin (H&E), and analyzed by a pathologist who was blinded for group identity. To score lung injury we used a modified VILI histology scoring system as previously described [14]. In short, four pathologic parameters were scored on a scale of 0 – 4: (a) alveolar congestion, (b) hemorrhage, (c) leukocyte infiltration, and (d) thickness of alveolar wall/hyaline membranes. A score of 0 represents normal lungs; 1, mild, < 25% lung involvement; 2, moderate, 25 – 50% lung involvement; 3, severe, 50 – 75% lung involvement and 4, very severe, > 75% lung involvement. The total histology score was expressed as the sum of the score for all parameters.

Assays

Total protein levels in BALF were determined using a Bradford Protein Assay Kit (OZ Biosciences, Marseille, France) according to manufacturers’ instructions with bovine serum albumin as standard. Cytokine and chemokine levels in lung homogenates were measured by enzyme–linked immunosorbent assay (ELISA) according to the manufacturer’s instructions. Tumor necrosis factor (TNF)–α, interleukin (IL)–6, macrophage inflammatory protein (MIP)–2 and keratinocyte–derived chemokine (KC) assays were all obtained from R&D Systems (Abingdon, UK). Thrombin–antithrombin complex levels in BALF were measured with a mouse specific ELISA as previously described [15].

Statistical analysis

All data in the results are expressed as means ± SD or median ± interquartile range, where appropriate. To detect differences between groups, the Dunnett method or Mann Whitney U test, in conjunction with two–way analysis of variance, was used. A p value of < .05 was considered significantly. All statistical analyses were carried out using SPSS 12.0.2 (SPSS, Chicago, IL).
Results

Hemodynamic and ventilator monitoring

All instrumented animals survived 5 hours of MV after which they were sacrificed. Control animals survived anesthesia and were also sacrificed after 5 hours. Hemodynamic monitoring demonstrated stable conditions throughout the experiment (figure 1): systolic blood pressure and heart rate remained stable in all animals for the complete duration of MV, with no differences between WT and TF+/- mice.

![Figure 1](image)

Figure 1 Hemodynamic parameters during 5 hours of mechanical ventilation: Systolic blood pressure (A) and heart rate (B, beats/min) were measured at three time points (t = 0, 2.5 and 5 hours after start of mechanical ventilation) in mice ventilated with low tidal volumes (Low Vt) and high tidal volumes (High Vt). Each mentioned group consisted of heterozygote TF knockout (TF+/-) mice and their wild-type (WT) littermates. Data represent means of 12 mice.

Blood gas analysis from LV—mice and HV—mice are demonstrated in table 1. No differences were observed between the different parameters of blood gas analysis, except from arterial oxygen tension (PaO₂). PaO₂ was significantly higher in HV—mice as compared to LV—mice.

| Table 1 Arterial blood gas analysis after 5 hours of mechanical ventilation |
|-------------------------------------------------------------|-----------------|-----------------|-----------------|-----------------|
|                  | Low Vt | TF+/- | WT   | TF+/- |
| pH               | 7.45 (0.06) | 7.44 (0.08) | 7.44 (0.08) | 7.44 (0.07) |
| PaCO₂ (mmHg)     | 33.9 (7.8)   | 35.0 (9.6)   | 32.5 (7.8)   | 33.2 (7.5)   |
| PaO₂ (mmHg)      | 172 (48)     | 165 (39)     | 234 (26)*    | 236 (24)*    |
| HCO₃⁻ (mmol/l)   | 22.6 (2.3)   | 22.7 (2.9)   | 21.1 (1.9)   | 22.5 (2.1)   |
| BE               | -0.5 (0.7)    | 0.8 (2.7)    | -2.1 (1.8)   | 0.8 (2.1)    |

Data represent mean (SD); Low Vt, mice ventilated for 5 h with a Vt of 7.5 ml/kg; High Vt, mice ventilated for 5 h with a Vt of 15 ml/kg. WT, wild-type mice; TF+/-, heterozygous TF knockout mice; n = 6 per group. *p < .05 vs. low Vt, mice; †p < .01 vs. low Vt, mice.
Lung injury

Lung W/D ratio was significantly higher with both MV–strategies compared with controls (p = .015 in LV–mice and p < .001 in HV–mice). W/D ratio in HV–mice was higher as compared to LV–mice (p = .039). No differences were seen between WT and TF+/- mice (figure 2A).

![Figure 2](image)

**Figure 2** Wet/dry (W/D) ratios of the left lung (A), total protein level in BALF (B) and number of neutrophils in BALF (C) after 5 hours in control (C) mice, mice ventilated with low tidal volumes (LV) and high tidal volumes (HV). Each mentioned group consisted of heterozygote TF knockout (TF+/−) mice and their wild-type (WT) littermates. Data represent means ± SD (A) or median and interquartile range (B and C) of six mice.

In accordance, total BALF protein levels were significantly higher in both MV–strategies as compared to controls (p < .001 for LV and HV mice). There was also a significant difference between HV and LV–mice (p < .001). No differences were seen between WT and TF+/- mice (figure 2B).

The number of neutrophils in BALF was significantly higher in HV–mice as compared to control (p < .001). There was a trend for more neutrophils in LV–mice as compared to control (p = .059). No differences were seen between WT and TF+/- mice (figure 2C).

The histopathological changes were minor. None of the lungs showed signs of inflammation, congestion or hyaline membranes. Only hemorrhage could be seen. The pulmonary histopathology score was higher in HV–mice as compared to control (p < .001). There was also a significant difference between LV and HV–mice (p = .001). No difference in pulmonary hemorrhage was seen between WT and TF+/- mice.

**Pulmonary coagulopathy**

TATc–levels in BALF were significantly higher in HV–mice as compared to control (p < .001). There was a trend for higher levels of TATc in LV–mice as compared to control (p = .051). There was also a significant difference between LV and HV–mice (p = .006). TF+/-
mice demonstrated lower levels of TATc in both ventilation groups (p = .04 in LVr-mice and p = .0026 in HVr-mice; figure 3).

**Figure 3** Thrombin-antithrombin complexes (TATc) levels in bronchoalveolar lavage fluid after 5 hours of mechanical ventilation in control (C) mice, mice ventilated with low tidal volumes (LVr) and high tidal volumes (HVr). Each mentioned group consisted of heterozygote TF knockout (TF+/-) mice and their wild-type (WT) littermates. Data represent median and interquartile range of six mice. *p < .05 vs. WT

**Pulmonary inflammation**
Mechanically ventilated mice demonstrated higher pulmonary levels of TNF–α and IL–6 as compared to control mice (p < .001 for LVr and HVr-mice). For both cytokines there was also a significant difference between LVr and HVr-mice (p < .001). TF+/- mice demonstrated higher levels of IL–6 in both ventilation groups as compared to WT mice (p = .002 for LVr and HVr mice). These heterozygous TF mice also demonstrated higher pulmonary levels of TNF–α in HVr-mice (p = .002; Figure 4A and 4B). Mechanically ventilated mice demonstrated higher pulmonary levels of MIP–2 and KC as compared to control (p < .001 for LVr and HVr-mice). For MIP–2 there was also a significant difference between HVr and LVr mice (p < .001). TF+/- mice demonstrated higher levels of MIP-2 and KC in LVr mice (p = .002). These heterozygous TF mice also demonstrated higher pulmonary levels of KC in HVr-mice (p = .015; Figure 4C and 4D).

**Systemic inflammation**
Systemic levels of IL–6 and KC were elevated in both ventilation groups as compared to control (p < .001 for both LVr and HVr mice). No differences were seen between TF+/- and WT mice (figure 5).
Figure 4 Pulmonary levels of interleukin (IL)-6 (A), tumor necrosis factor (TNF)-α (B), macrophage inflammatory protein (MIP)-2 (C) and keratinocyte-derived chemokine (KC) (D) in lung tissue homogenate after 5 hours in control (C) mice, mice ventilated with low tidal volumes (LVt) and high tidal volumes (HVt). Each mentioned group consisted of heterozygote TF knockout (TF+/-) mice and their wild-type (WT) littermates. Data represent median and interquartile range of six mice. *p < .05 vs. WT; †p < .01 vs. WT.

Figure 5 Systemic levels of interleukin (IL)-6 (A) and keratinocyte-derived chemokine (KC) (B) in plasma after 5 hours in control (C) mice, mice ventilated with low tidal volumes (LVt) and high tidal volumes (HVt). Each mentioned group consisted of heterozygote TF knockout (TF+/-) mice and their wild-type (WT) littermates. Data represent median and interquartile range of six mice. The levels of IL-6 and KC in control mice were below the detection limit of the assay.
Discussion

There is mounting evidence indicating that TF is important for a procoagulant response in the pulmonary compartment. We here show that TF contributes to ventilator-induced coagulopathy in a mice model of VILI. Although relative TF deficiency attenuated ventilator-induced coagulopathy, it does not reduce VILI. In respect to some endpoints of VILI, relative TF deficiency even induces more inflammation.

TF inhibition by means of antibodies has been shown to reduce lung inflammation and injury in experimental endotoxemia and bacteremia [16,17]. Because of extensive cross-talk between coagulation and inflammation, causing reciprocal activation and amplification, one may expect less pulmonary inflammation in case of TF inhibition. However, a number of studies in pulmonary and non-pulmonary models show that inhibition of the TF-FVII pathway does not reduce inflammation. In an Escherichia coli peritonitis mice model, treatment with rNAPc2, a potent and selective small protein inhibitor of the TF-FVIIa pathway, attenuated the local procoagulant response, but not the inflammatory response [18]. Moreover, in a Streptococcus pneumoniae pneumonia model in mice, rNAPc2 treatment attenuated the procoagulant response, but increased chemokine levels of KC and MIP-2 compared to control animals (that received only placebo), without influencing clearance of bacteria [19]. Our results in the VILI model are in agreement with these studies, showing higher levels of cytokines and chemokines in TF+/- mice as compared to WT mice. In line with this are the results from human volunteer studies, in which a low dose lipopolysacharide was administered. Intravenous infusion of TF pathway inhibitor (TFPI) attenuates activation of coagulation, but did not influence inflammatory pathways [20,21].

We were not able to confirm our results in this study using a direct TF–pathway blocker, to see if effects were similar as in heterozygous TF+/- mice. An anti-TF antibody would block TF activity probably more than TF activity of TF+/- mice, which is about 50% of that of WT mice [13]. Another, abovementioned TF–pathway blocker, rNAPc2, inhibits TF-FVIIa-mediated coagulation by a mechanism of action that differs from that of the physiological inhibitor of TF, tissue factor pathway inhibitor (TFPI). Unfortunately, rNAPc2 and TFPI are presently unavailable.

Our results indicate that TF plays a role in ventilator-induced coagulopathy and that inactivation of the TF-pathway could lead to less coagulation and less fibrin deposition in the airways during mechanical ventilation. On the other hand, less TF activity, leads to more pulmonary inflammation in our VILI model. The question remains whether pulmonary inflammation is a harmful process or an adaptive mechanism, protecting the host from lung injury (as reflected by W/D ratio, total protein level in BALF or histopathology score).
Our model has several limitations. First, lung-protective mechanical ventilation with LV\(_T\) may promote development of atelectasis. In our model we used deep inflation every 30 minutes to prevent atelectasis. Periodic deep inflation can open the lung, but if delivered too frequently may cause damage via repeated overdistension by itself [22]. It may be speculated that the lungs were not recruited frequently enough to prevent atelectasis in this study. Indeed, HV\(_T\)-mice demonstrated higher PaO\(_2\) levels than LV\(_T\)-mice. Some pulmonary inflammation seen in LV\(_T\)-mice may have been caused by atelectotrauma. Alternatively, even mechanical ventilation using a V\(_T\) of 7.5 ml/kg may still cause overdistension of alveoli. Second, the V\(_T\) used in HV\(_T\)-mice are quite large. Although there is still underuse of lung-protective ventilation with the use of lower V\(_T\) [23,24], V\(_T\) have declined gradually over the past 10 years [25,26]. However, V\(_T\) of as large as 15 ml/kg are reported to be used in human clinical studies [27,28]. Therefore our comparison may still reveal relevant information on lung injury caused by mechanical ventilation.

In conclusion, TF seems to play an important role in the pathogenesis of disturbed alveolar fibrin turnover during mechanical ventilation. However, relative TF-deficiency does not reduce VILI. In respect to some endpoints of VILI, relative TF-deficiency even seems to induce more inflammation. Further studies are warranted to establish the effect of TF inhibition by drugs on ventilator-induced coagulopathy and VILI.
The Role of Tissue Factor in Ventilator–Induced Pulmonary Coagulopathy in Mice

References


