Lung protective mechanical ventilation
Wolthuis, E.K.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 13

Recombinant Human Soluble Tumor Necrosis Factor–alpha Receptor Fusion Protein Partly Attenuates Ventilator–Induced Lung Injury


Shock 2008 [accepted for publication]
Abstract

**Background:** Ventilator–induced lung injury (VILI) is mediated, at least in part, by tumor necrosis factor–α (TNF). We determined the effect of a recombinant human soluble TNF receptor fusion protein (etanercept) on mechanical ventilation (MV)–induced changes in a murine VILI–model.

**Methods:** After pretreatment with etanercept or placebo, C57Bl/6 mice were anesthetized and randomized to MV with either low tidal volumes (VT, ~ 7.5 ml/kg) or high VT (~ 15 ml/kg) for 5 hours. Instrumented but spontaneously breathing mice served as controls.

**Results:** MV resulted in VILI in both ventilation groups: ventilated animals showed higher lung histopathology scores, higher protein levels in bronchoalveolar lavage fluid (BALF), more neutrophil cells in BALF, higher thrombin–antithrombin complex (TATc)–levels in BALF and higher pulmonary cytokine levels as compared to controls. More injury was found with large VT. Wet to dry (W/D) ratio was only increased in high VT mice as compared to control. The number of caspase 3 positive cells was not significantly different among the three groups. Etanercept treatment reduced the number of neutrophil in BALF, as well as pulmonary cytokine levels. W/D–ratios, histopathology scores and local protein BALF levels were not influenced by etanercept treatment. The number of caspase 3 positive cells was significantly higher after administration of etanercept.

**Conclusion:** Inhibition of TNF by etanercept attenuates VILI–associated inflammatory changes and ventilator–induced coagulopathy.
**Introduction**

Mechanical ventilation (MV) aggravates lung damage in patients with acute lung injury and may even initiate pulmonary biotrauma, referred to as “ventilator–induced lung injury” (VILI) in experimental models of mechanical ventilation or “ventilator–associated lung injury” (VALI) in humans [1,2]. Biotrauma occurs in the absence of gross structural lung damage. The biotrauma hypothesis proposes that biophysical forces are responsible for alteration of normal cellular physiology in the lungs leading to a proinflammatory milieu [3], disturbances in alveolar fibrin turnover [4,5], and changes in pulmonary repair, remodeling, and mechanisms involving programmed cell death.

MV can damage pulmonary epithelium through either apoptotic or non–apoptotic cell death. Apoptosis is a form of regulated cell death in which activation of specific intracellular serine rich proteases (caspases) leads to DNA cleavage and cell death. In *in vitro* experiments, mechanical strain induced pro–apoptotic changes in human lung epithelial cells [6,7]. Furthermore, *in vivo* animal experiments demonstrated that impairment of apoptosis pathways limits pulmonary inflammation, lung injury, and protected against multiple organ failure and death [8,9]. Therefore, it has been proposed that intra–alveolar apoptosis is a potentially harmful process that could be targeted in the treatment of (ventilator–associated) lung injury [10].

VILI possesses a significant tumor necrosis factor–α (TNF)–dependent component [11], including the optional capacity to induce apoptosis. Also, neutrophil recruitment is substantially attenuated in TNF–receptor knock–out mice and mice treated with intratracheally administered anti–TNF antibodies [11]. Etanercept is a humanized dimeric fusion protein consisting of the extracellular ligand–binding portion of the 75–kDa TNF receptor linked to the Fc portion of IgG1. It can bind to two TNF molecules blocking their interaction with cell surface TNF receptors, thereby interfering with biological activity of TNF. TNF inactivation by etanercept is a thousand times stronger than TNF inactivation by p75 monomeric TNF receptor [12]. Etanercept has been proven to inhibit the activity of TNF in several animal model systems of inflammatory and autoimmune diseases [13]. It has been tested in numerous clinical trials and it has been approved for rheumatic disorders.

Present strategies aiming at minimizing VALI in the critically ill patients consist of using low tidal volumes (VT) [14]. However, additional strategies to attenuate pulmonary inflammation may be useful to further reduce VALI. The aim of the present investigation was to determine the effect of etanercept on VILI, using low and high VT.
Materials and Methods

The Animal Care and Use Committee of the Academic Medical Center approved all experiments. Animal procedures were carried out in compliance with Institutional Standards for Human Care and Use of Laboratory Animals.

Animals

Female C57Bl/6 mice (6 – 8 week old, 17 – 20 gram, Charles River, Maastricht, the Netherlands) were maintained at the animal care facility of the Academic Medical Center according to institutional guidelines. Control animals (n = 12) served as non-ventilated controls. The other animals were all mechanically ventilated for 5 h with 2 different MV-strategies and 2 different pre-treatments (either placebo or etanercept). Thus, 6 groups of animals were formed (total n = 72). Half of these mice were used for broncho-alveolar lavage (BAL, right lung) and wet to dry ratio (W/D; left lung). 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).

Anesthesia protocol

Mice received an intraperitoneal (i.p.) bolus of 1 ml 0.9% saline 1 hour before start of anesthesia and initiation of MV. A tracheostomy (Y-tube connector, 1.0 mm outer diameter, 0.6 mm inner diameter) (VBM Medizintechnik GmbH, Germany) was inserted under general anesthesia with i.p. 126 mg/kg ketamine, 0.2 mg/kg medetomidine and 0.5 mg/kg atropine. Body temperature was kept constant at 36.5 – 37.5°C with the use of rectal temperature monitoring and a warming device. Maintenance anesthesia consisted of 36 mg/kg ketamine, 0.04 mg/kg medetomidine and 0.075 mg/kg atropine. Maintenance-mix was administered via an intraperitoneal catheter (PE 10 tubing, BD, Breda, the Netherlands) every hour. To correct for hypovolemia sodium bicarbonate was administered via the intraperitoneal catheter every 30 minutes.

Mechanical ventilation

Mice were placed in a supine position and connected to a human ventilator (Servo 900 C, Siemens, Solna, Sweden). Pressure controlled MV was initiated with either an inspiratory pressure of 10 cmH2O (resulting in Vt ~ 7.5 ml/kg; low Vt) or an inspiratory pressure of 18 cmH2O (resulting in Vt ~ 15 ml/kg; high Vt). The respiratory rate was set at 120 breaths/min with low Vt and 70 breaths/min with large Vt, respectively. Positive end expiratory pressure (PEEP) was set at 2 cmH2O in both MV–strategies. 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 to recruit atelectatic lung tissue. FiO2 was set at 0.5. Control mice, receiving half the dose of induction anesthesia, were instrumented, but not ventilated.
**Hemodynamic and ventilatory monitoring**

A subset of mice were used for monitoring of non-invasive blood pressure and pulse every hour during the five hour period of MV using a murine blood pressure/pulse monitor (Visitech Systems, Apex, North Carolina, USA). $V_t$ was monitored hourly with a specially designed mice Fleisch–tube connected to the body–plethysmograph. 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, Akron, OH) and stored on a computer for post acquisition off line analysis. A minimum of 5 consecutive breaths was selected for analysis of the digitized VT signals. After 5 h of MV arterial blood from the carotid artery was taken for blood gas analysis.

**Etanercept**

To neutralize TNF, 100 μg etanercept (Enbrel Wyeth Pharmaceuticals BV, Hoofddorp, the Netherlands) was administered i.p. 15 hours and 1 hour before start of MV. The dose of 100 μg had been shown to be efficient to neutralize TNF [15-18]. Etanercept was reconstituted at a concentration of 25 mg/ml in sterile water as suggested by the manufacturer and was further diluted in PBS to a final concentration of 500 μg/ml. Control mice received the same volume of sterile saline i.p.

**Bronchoalveolar lavage**

BAL was performed by instilling three times 0.5 ml aliquots of saline by a 22–gauge Abbocath–T catheter (Abbott, Sligo, Ireland) into the trachea. Approximately, 1.2 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 Giemsa–stained citospin preparations. Cell–free supernatants were stored at –20°C.

**Wet/dry–ratio**

Lungs were weighed and subsequently dried for three days in a 65°C stove. The ratio of wet weight to dry weight represents tissue edema.

**Lung homogenates**

Lungs were homogenized in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). Lung homogenates were diluted 1:1 in lysis buffer (150 mM NaCl, 15 mM Tris, 1 mM MgCl₂·H₂O, 1mM CaCl₂, 1% Triton X-100, 100 μg/mL pepstatin A, leupeptin and aprotinin, pH 7.4) and incubated at 4°C for 30 min. Cell-free supernatants were obtained by centrifugation at 1500 x g for 15 min and stored at –20°C.
Histopathology

Lungs were fixed in 4% formalin and embedded in paraffin. Four μm sections were stained with hematoxylin-eosin, 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 [19]. 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

BAL–fluid (F) total protein levels were determined using a Bradford Protein Assay Kit (OZ Biosciences, Marseille, France) according to manufacturers’ instructions. TATc was measured with a mouse specific ELISA [20]. Cytokine and chemokine levels in lung homogenates were measured by ELISA according to the manufacturer’s instructions. TNF, interleukin (IL)–6, macrophage inflammatory protein (MIP)–2 and keratinocyte chemoattractant (KC) assays were all obtained from R&D Systems (Abingdon, UK).

Caspase 3 immunohistochemistry

Activated caspase 3, a distal enzyme in the caspase cascade, can be detected in cell and tissues using antibodies specific for (cleaved) activated form of caspase 3. In short, deparaffinized slides were boiled in citrate buffer (pH 6.0). After blocking of non-specific binding and endogenous peroxidase activity, slides were incubated with rabbit anti-human active caspase 3 polyclonal antibody (Cell Signaling, Beverly, MA) followed by biotinylated swine anti-rabbit antibody (Dako) [21]. At a magnification of 400 x, we counted the number of caspase 3 positive cells in 10 randomly chosen fields. The total number of caspase 3 positive cells was the summation of caspase 3 positive cells in 10 fields.

Statistical analysis

All data are expressed as means (SEM) or median (IQR), depending on normal distribution of data or not. To detect differences 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 statistically significant. All statistical analyses were carried out using SPSS 12.0.2 (SPSS, Chicago, IL).
Results

Physiological measurements

Body temperature was strictly controlled between 36.5 – 37.5°C in all experiments, to achieve normal homeostasis during prolonged procedures. Systolic blood pressure was well maintained over the 5 hour MV period in the low as well as in the large VT group in both placebo and treatment mice. Blood gas parameters were also maintained within physiological range and were not different among groups (data not shown).

Lung injury with low and large VT

W/D ratio was higher in high VT mice as compared to low VT or control mice (p < .001; figure 1A). In accordance, pulmonary histopathology score was higher in ventilated mice (3.0 [0.8] for low VT and 3.5 [1.0] for high VT) as compared to control mice (0.0 [0.0], p < 0.05). Total BALF–protein levels were significantly higher in high VT mice (309 [278 - 392] μg/ml) as compared to low VT mice (189 [124 - 276] μg/ml, p < .001) and control mice (89 [70 - 102] μg/ml, p < 0.01; figure 1B).

![Figure 1](https://via.placeholder.com/150)

Figure 1 W/D ratios of the left lung (A), total protein level in BALF (B) and number of neutrophils in BALF (C) in control (C), low VT and high VT mice. White boxes represent the placebo group and grey boxes represent the etanercept group. Data represent means (SEM) or median (IQR) of 6 mice. †p < .01 vs. placebo.

The total number of cells in the BALF was not influenced by either MV–strategy. However, the number of neutrophils was significantly higher in high VT mice (15.1 [9.6 – 20.6] x 104/ml) as compared to low VT mice (5.5 [1.9 – 7.1] x 104/ml; p < .001) and control mice (0 [0 – 1.0] x 104/ml, p < 0.001; figure 1C).

Pulmonary levels of TNF, IL-6, MIP-2 and KC were influenced by MV, with higher levels in the high VT mice. Levels of TNF were significantly higher in both ventilated groups as compared to control mice (p < .001). There was also a difference between low and high VT mice (p = .014). Pulmonary IL-6 levels were significantly higher in both ventilated groups as compared to control (p = .01 for low VT and p < .001 for high VT; figure 2A). Levels of IL-
6 were also significantly higher in high VT mice as compared to low VT mice (p < .001). Concentrations of MIP-2 and KC were higher in both ventilated groups as compared to control mice (p < .001; figure 2B and 2C). In high VT mice levels of both chemokines were also significantly higher as compared to low VT mice (p < .001).

TATc in BALF were significantly higher in high VT mice (17.3 [16.7 – 24.3] ng/ml) as compared to low VT mice (2.6 [1.6 – 6.5] ng/ml, p < .001) and non–ventilated controls (0.29 [0.23 – 0.82] ng/ml, p < 0.001; figure 3).

The mean number of caspase 3 positive cells was 13.9 (4.2) in high VT mice as compared to 5.3 (1.1) in low VT mice and 2.3 (0.6) in controls (figure 4). The number of caspase 3 positive cells was not significantly different among the three groups.

**Effect of etanercept on VILI**

Treatment with etanercept did not influence the W/D ratios (figure 1A), nor the pulmonary histopathology score. Treatment with etanercept did not influence the protein level in BALF (figure 1B).

In the low VT mice administration of etanercept attenuated cell influx into the lung from 59.4 [29.1 – 87.6] x 10^4/ml to 14.0 [11.8 – 24.8] x 10^4/ml (p = .011). In both ventilated groups (low and high VT) the number of neutrophils were significantly lower after administration of etanercept (figure 1C). In low VT mice the number of neutrophils declined from 5.5 [1.9 – 7.1] x 10^4/ml to 0.6 [0.1 – 1.9] x 10^4/ml BALF. In high VT mice neutrophils decreased from 15.1 [9.6 – 20.6] x 10^4/ml to 2.7 [1.5 – 7.5] x 10^4/ml BALF.
Pulmonary levels of IL-6, MIP-2 and KC were all significantly lower in both ventilated groups after etanercept treatment (figure 2). After administration of etanercept TATc levels decreased significantly in large VT mice (figure 3). Etanercept treatment induced an increase in the number of caspase 3 positive cells in control and low VT mice (figure 4).

Discussion
In a murine model of MV we found that both physiologic and high VT induced VILI, as demonstrated by an elevated protein level in BALF and more lung injury on histopathological examination when compared to non–ventilated controls. Pulmonary inflammation, as measured by the number of neutrophils in BALF and local cytokine levels, was also higher in ventilated animals as compared to non–ventilated controls as was pulmonary coagulation. Treatment with etanercept did not influence the W/D ratio, histopathology or total protein level in BALF. However, the number of neutrophils in both ventilation groups declined after etanercept treatment. Pulmonary levels of IL-6, MIP-2 and KC were also decreased after treatment with etanercept. In addition, administration of etanercept resulted in less pulmonary coagulation in high VT mice. Etanercept exerted pro-apoptotic effects in the lung.
Our model has some limitations. First, lung-protective MV with low $V_T$ may promote atelectasis. Low $V_T$ employed in the present study is equal to that of spontaneously breathing C57Bl/6 mice (6 – 9 mL/kg) [22]. In our model we used deep inflation every 30 minutes to prevent atelectasis. Allen et al. demonstrated that periodic recruitment with relatively frequent deep inflations during ventilation with low $V_T$ can improve oxygenation, ventilation, and lung mechanical function with no evidence of lung injury by 2 h in mechanically ventilated mice [23]. They suggest a threshold tolerance of deep inflation frequency, beyond which the intrinsic reparative properties of the lung epithelium are overwhelmed. Some pulmonary inflammation seen in low $V_T$ mice therefore may be caused by atelectotrauma. Alternatively, even MV using a tidal volume of 7.5 ml/kg may still cause overdistension of alveoli. Second, the $V_T$ used in high $V_T$ mice are quite large. Although there is still underuse of low $V_T$ ventilation, $V_T$ have declined gradually over the past 10 years. However, $V_T$ of as large as 15 ml/kg are reported to be used in human clinical studies [24,25]. Therefore our comparison may still reveal relevant information on lung injury caused by MV.

Wilson et al. demonstrated that pulmonary neutrophil recruitment in response to high-stretch ventilation in the absence of underlying lung injury involves a significant TNF-mediated component [11]. Intratracheal administration of anti–TNF antibody and the use of TNF receptor knockout animals resulted in a decrease of alveolar neutrophil recruitment. Imai and coworkers showed that pretreatment with intratracheal administration of anti–TNF antibody attenuated VILI in a saline-lavaged rabbit model [26].
Our data are in agreement with these previous studies, demonstrating a decline in the number of neutrophils after treatment with etanercept, with a concomitant decline in the pulmonary cytokine/chemokine level. Clinical studies have provided convincing evidence that high $V_t$ ventilation can lead to an increase in production of TNF, locally and systemically. Ranieri et al. demonstrated that conventional ventilation, using $V_t$ of $\sim 11$ ml/kg and PEEP of 7 cmH$_2$O, causes an increase in TNF locally and systemically over 36 hours in patients with established ARDS [27]. A more protective ventilation strategy ($V_t$ $\sim 8$ ml/kg and 15 cmH$_2$O PEEP) resulted in less polymorphonuclear cell influx into the lungs, and lower TNF levels in BALF.

In our study some endpoints of lung injury (W/D–ratios, histopathology and local protein levels in BALF) were not influenced by administration of etanercept. These findings are in line with the results from experiments by Wilson et al., in which TNF double receptor (TNFR1 and TNFR2) knock out mice had the same protein level in BALF after either high or low stretch as compared to wild type animals, indicating a comparable degree of lung injury and pulmonary edema [11].

In our study we showed that etanercept had pro-apoptotic effects in the lung. In other inflammatory conditions, etanercept may limit the inflammatory response by the induction of apoptosis. In patients with Crohn’s disease, induction of apoptosis of activated inflammatory cells has been proposed as a mechanism of the protective effect of blocking TNF activity [29].

Etanercept has been tested in numerous clinical trials and it has been approved for treatment of rheumatoid arthritis, juvenile rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis and plaque psoriasis [30]. Lately, it has been reported that etanercept blocks the acute inflammatory cell response in an in vivo whole muscle autograft in mice [16]. Furthermore, in a murine model of pancreatitis induced by cerulein, etanercept attenuated all aspects of pancreatitis [31]. This new drug is now being evaluated for treating many other clinical disorders, in which up-regulation of TNF appears to play a pathophysiological role. Our data suggest etanercept may be of value in patients at risk for VALI.

In conclusion we found that a recombinant human soluble TNF receptor fusion protein (etanercept) attenuates, in part, inflammatory and procoagulant changes caused by MV. Blockade of TNF signaling may have therapeutic potential to reduce pulmonary inflammation in patients. Further investigations are necessary to prove this hypothesis.
References


