Ventilator-induced lung injury:
pathogenesis & therapeutic interventions

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Learn from yesterday, live for today, hope for tomorrow.
The important thing is not to stop questioning.

– Albert Einstein

CONTENTS

CHAPTER

1

General introduction

Mechanical ventilation has become a standard technique to support life of the critically ill patient in the intensive care unit (ICU) [1,2]. In general, mechanical ventilation is applied when the patient's spontaneous ventilation is inadequate to maintain life. Especially patients who have developed acute respiratory failure require mechanical ventilation [3]. Patients diagnosed with acute lung injury (ALI) suffer from severe pulmonary dysfunction which may persist for a long period of time. The extent and severity of ALI differs among patients, with the acute respiratory distress syndrome (ARDS) being the most severe manifestation of this lung disease [4]. ALI and ARDS are characterized by the acute onset of diffuse neutrophilic alveolar infiltrates, protein-rich edema due to enhanced alveolar-capillary permeability and hypoxemic respiratory failure (ratio of partial pressure arterial oxygen and fraction inspired oxygen, $PaO_2/FiO_2 < 300$ for ALI or < 200 for ARDS) [5,6]. These pulmonary disorders may result from local injuries like pneumonia, gastric aspiration, near-drowning and lung contusion but also from systemic events such as severe sepsis, shock and blood transfusions [7,8].

At present, it is hypothesized that an inflammatory response in the lung - i.e. cytokine expression, granulocyte infiltration, etc - may precede pulmonary injury in the pathogenesis of ALI/ARDS [4,9]. Activated granulocytes are recognized to cause oxidative stress and protease activity in the alveoli, thereby inducing severe damage to pulmonary epithelial-endothelial barriers and leading to impaired gas exchange [10-12]. As a consequence, ALI/ARDS patients with inhomogeneously-injured, inflamed lungs will require mechanical ventilation with high pressures or tidal volumes to adequately oxygenate vital organs [13,14].

LOCAL EFFECTS OF MECHANICAL VENTILATION

Although mechanical ventilation is a life-saving procedure in the ICU, it may exacerbate or even initiate damage to the lung itself [15,16]. The pulmonary complications secondary to mechanical ventilation are known as ventilator-induced lung injury (VILI). Important features of VILI are production of inflammatory mediators, enhanced alveolar-capillary permeability, accumulation of protein-rich pulmonary edema and ultimately impaired gas exchange [17]. These patterns of injury are very similar to those seen in ALI/ARDS [18].

Possible underlying mechanisms of VILI

Various experimental models have been used to obtain further insight into the mechanisms underlying VILI. These *in vitro* and *in vivo* studies support the clinical observations that application of mechanical ventilation may lead to stretch trauma due to cyclic opening and closing and/or overdistention of alveoli [19,20]. Ventilator-induced cyclic opening and closing of alveoli results in enhanced local shear forces thereby leading to depletion of surfactant, a surface tension lowering substance, and rendering the lung more prone to collapse [21,22].

Extensive research demonstrated that surfactant depletion initiates loss of alveolar-capillary barrier function [23-26]. Due to increased epithelial-endothelial membrane permeability, serum proteins will leak from the blood circulation into pulmonary tissue. Protein accumulation in the lung will lead to surfactant inactivation, causing even more protein leakage and edema formation and eliciting a vicious circle of progressive lung injury [23,27].

Already in the 1940s, investigators recognized that conventional ventilation strategies with high pressures or tidal volumes cause overdistention of lung tissue and subsequently result in VILI [28,29]. Unfortunately, high pressures or tidal volumes cannot always be avoided when ventilating critically ill patients with injured, less compliant lungs. Recent research, however, showed that "lung-protective" ventilation strategies with low pressures or tidal volumes, which preserve alveolar integrity, may provoke the development of VILI as well [30-32].

Inflammation

Kawano et al. were the first to show that granulocyte depletion prior to injurious mechanical ventilation attenuated pulmonary dysfunction in an experimental model of surfactant deficiency, stressing the importance of inflammatory mediators in the pathogenesis of VILI [33]. Later, Tremblay et al. revealed that alveolar stretch imposed by *ex vivo* mechanical ventilation caused interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, macrophage-inflammatory protein (MIP)-2, interferon (IFN)-γ and IL-10 expression. These authors introduced the term "biotrauma" to describe the ventilator-induced secretion of inflammatory mediators (figure 1) [34,35]. In line with this concept, it has been shown that alveolar macrophages secrete cytokines and chemokines upon *in vitro* applied mechanical stretch [36]. Although alveolar macrophages are considered to be the most important source of pulmonary cytokines/chemokines, other cell types like fibroblasts, leukocytes, epithelial and endothelial cells are also capable of producing inflammatory mediators [37]. Clinical evidence for the biotrauma-hypothesis was given by Ranieri et al. [38]. In a randomized controlled trial, they observed elevated concentrations of IL-1β, TNF-α, IL-6 and IL-1 receptor antagonist in the bronchoalveolar lavage fluid (BALf) of ALI/ARDS patients ventilated with excessive tidal volumes.

At present, it is thought that the increased production of inflammatory mediators in the lung may induce or aggravate pulmonary injury [39-41]. Previous research suggested that enhanced pro-inflammation due to mechanical ventilation makes the patient more vulnerable to a "second hit" [42]. Importantly, mechanical ventilation itself may be the "second hit" when ventilating critically ill patients suffering from pulmonary injuries like ALI/ARDS or systemic events like sepsis [43-45].

Endothelial activation

It has been recognized that serious inflammatory diseases like VILI and ALI/ARDS induce a phenotypic shift in pulmonary endothelial cells, characterized by secretion of inflammatory

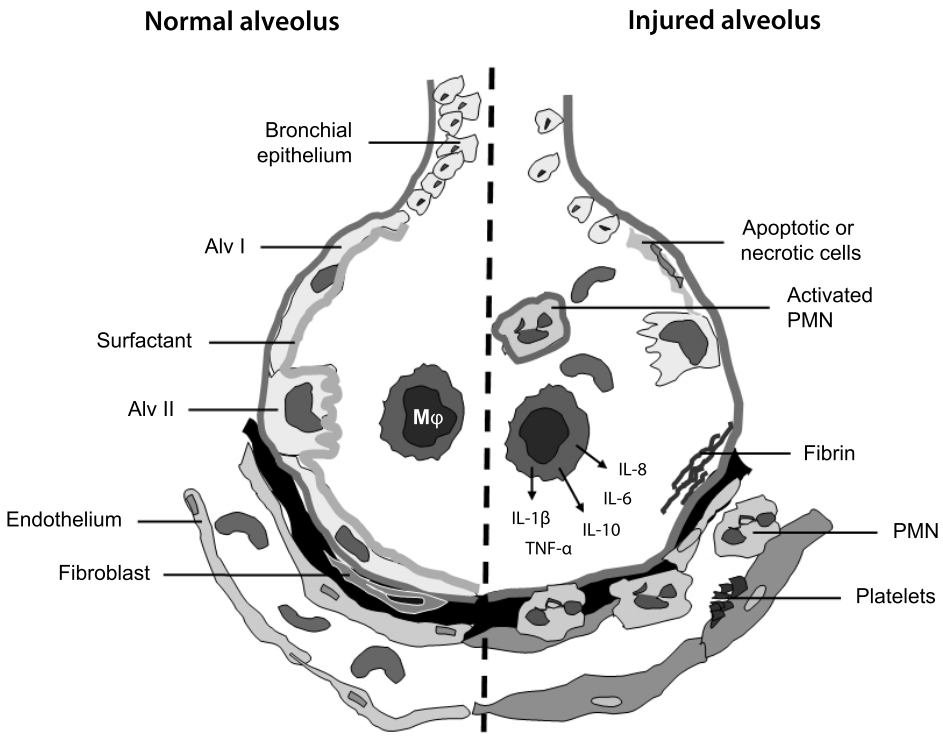

Figure 1: Ventilator-induced lung inflammation.
Alveolar stretch due to mechanical ventilation may induce or aggravate the release of multiple inflammatory mediators into the lung. These include both pro-inflammatory (IL-1β, TNF-α, IL-6 and IL-8) and anti-inflammatory (IL-10) mediators. Chemoattractants, such as IL-8 (or the rodent equivalents MIP-2 and KC), will recruit polymorphonuclear cells (PMNs) to the lung. The inflammatory process will lead to loss of alveolar-capillary barrier function, apoptotic and/or necrotic cell death and eventually formation of fibrin strands. Furthermore, surfactant will become inactivated and gas exchange impaired. Illustration adapted with permission from H.A.E. Vreugdenhil (Thesis "Mechanical ventilation and immune function" by H.A.E. Vreugdenhil 2003).
Alv I, Alv II = alveolar type I or II cells; IL = interleukin; KC = keratinocyte-derived chemokine; MIP = macrophage inflammatory protein; TNF = tumor necrosis factor.

and chemotactic mediators, expression of adhesion molecules and increased permeability [46]. In inflammation, the presence of the pro-inflammatory cytokines IL-1β and TNF-α leads to endothelial activation [47]. Subsequently, activated endothelial cells secrete chemotactic cytokines (chemokines) which are essential for the recruitment of leukocytes [48]. The main pulmonary chemoattractant is IL-8 or its rodent equivalents MIP-2 and keratinocyte-derived chemokine (KC) [49]. Although circulating leukocytes are attracted to the site of inflammation by chemoattractant gradients another mechanism is needed to halt the movement of leukocytes and enable them to exit blood vessels and infiltrate inflamed tissue, i.e. the adherence of leukocytes to vascular endothelium [47]. Besides release of chemokines and

other inflammatory mediators, activated endothelial cells express different types of adhesion molecules on their cell surface thereby directing the multi-step cascade of leukocyte adhesion and transmigration between endothelial cells. The initial step involves members from the selectin family (P- and E-selectin) which tether circulating leukocytes to vascular endothelium and facilitate rolling of leukocytes along the blood vessel wall [50]. Subsequent leukocyte adhesion and extravasation are mediated by the immunoglobulin (Ig) superfamily comprising vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1 and platelet-endothelial cell adhesion molecule (PECAM)-1. In affected tissue, activated leukocytes release oxygen-based free radicals and proteolytic enzymes to restore physiological conditions. However, the accumulation of these cells may cause varying degrees of tissue damage and organ dysfunction [10].

Previous research has demonstrated that leukocyte-endothelial interactions are important in the pathogenesis of severe inflammatory diseases related to VILI, like ALI/ARDS [10,51-53]. Gando et al. observed that soluble levels of P-selectin, E-selectin, ICAM-1 and VCAM-1 were elevated in blood plasma of ALI/ARDS patients within 24 hours after the diagnosis [52]. Furthermore, they showed a marked increase in these soluble adhesion molecules when subdividing the patients into survivors and non-survivors implying that adhesion molecules may have a prognostic value for the development and clinical outcome of ALI/ARDS. So, manipulating the course of endothelial activation by therapeutic interventions may be advantageous for the clinical outcome of ventilated, critically ill patients.

Alveolar-capillary permeability

The primary site of pulmonary gas exchange is the < 0.2 µm thin part of the alveolar-capillary membrane, consisting of alveolar epithelial and capillary endothelial cells [54,55]. Already in the 1980s, several investigators observed that mechanical ventilation and the subsequent stretch of pulmonary tissue induces damage to epithelial-endothelial barriers consequently impairing gas exchange [20,56,57]. In addition, Dreyfuss et al. demonstrated that enhanced microvascular permeability was responsible for the formation of pulmonary edema during mechanical ventilation with high tidal volumes [20]. Besides apoptotic and/or necrotic cell death, loss of alveolar epithelial and capillary endothelial cell integrity is thought to play a crucial role in the ventilator-induced disruption of alveolar-capillary barriers [58].

Apoptotic cell death

Apoptosis is distinguished by cell shrinkage and nuclear fragmentation, while cell organelles and the plasma membrane maintain their integrity for a prolonged period [59,60]. The apoptotic cell death cascade is mainly regulated and executed by cysteine aspartyl-specific proteases, caspases.

At present, it is known that two distinct routes are capable of initiating apoptosis, i.e. the intrinsic and extrinsic apoptotic pathway (figure 2) [61]. In response to oxidative stress, DNA

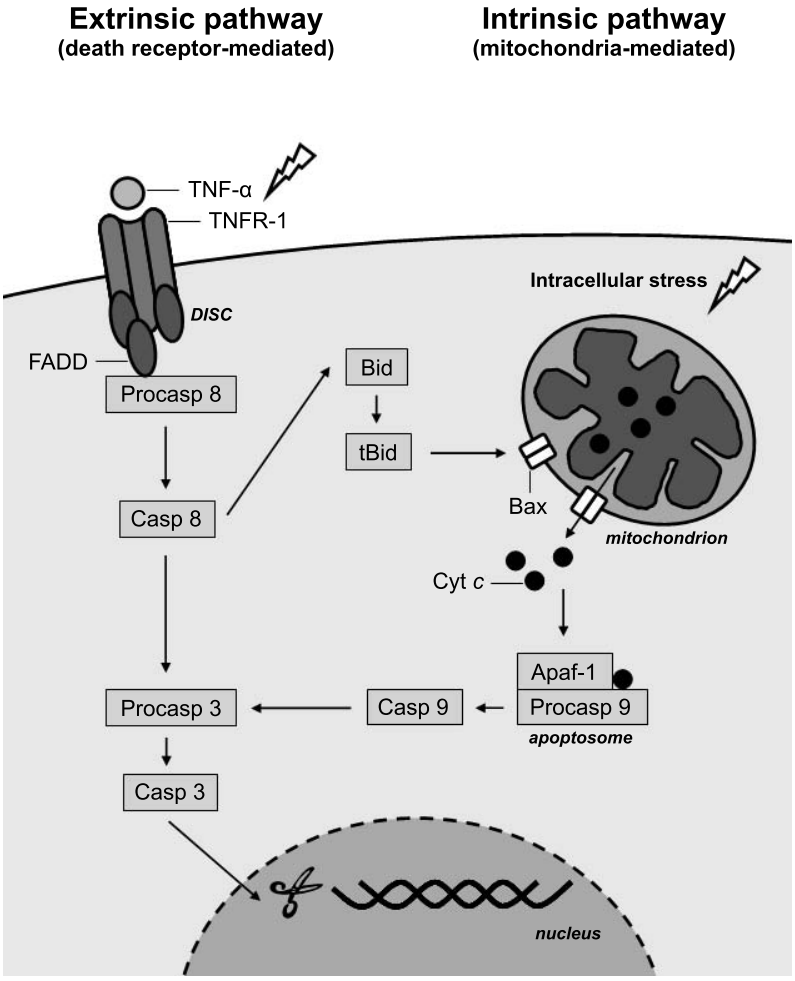

Figure 2: Two distinct pathways of apoptotic cell death.
Intrinsic (mitochondria-mediated) pathway: In response to severe intracellular stress, cytochrome *c* will be released from the mitochondria into the cytosol. Subsequently, cytochrome *c* forms an apoptosome complex together with apoptotic protease-activating factor (APAF)-1 and procaspase-9. In the apoptosome, procaspase-9 is cleaved to its mature form and will activate the downstream executioner caspases (mainly 3 and 7). Mature caspase-3 activates caspase-dependent DNAses leading to chromatin condensation and DNA degradation. *Extrinsic (death receptor-mediated) pathway:* Triggering of cell surface death receptors, like TNFR-1 and Fas, will cause formation of a death-inducing signaling complex (DISC). In the DISC, procaspase-8 (or 10) is cleaved to its mature form and subsequently released into the cytosol to activate important executioner caspases like 3, 6 and 7. Caspase-8 is also involved in an indirect pathway linking the extrinsic and intrinsic routes of apoptotic cell death. In this pathway, the activation of caspase-8 will lead to cleavage of Bid which in turn causes permeability of the mitochondrial membrane (Bax pore).
Casp = caspase (cysteine aspartyl-specific protease); Cyt *c* = cytochrome *c*; FADD = Fas-associated protein with death domain; Procasp = procaspase; (t)Bid = (truncated) BH-3 interacting domain death agonist; TNF = tumor necrosis factor; TNFR = TNF receptor.

damage or other types of severe intracellular stress, mitochondria undergo marked changes in membrane integrity and activate the intrinsic (mitochondria-mediated) pathway [62,63]. Enhanded mitochondrial membrane permeability leads to release of pro-apoptotic proteins, such as cytochrome *c*, into the cytosol. Once cytochrome *c* is released from the mitochondria it binds to apoptotic protease-activating factor (APAF)-1 and adenosine triphosphate (ATP), which subsequently tethers procaspase-9 to generate an apoptosome complex [64,65]. In the apoptosome, procaspase-9 is cleaved to its mature form and activates the downstream executioner caspases (mainly 3 and 7). Mature executioner caspases activate caspase-dependent DNAses thereby causing chromatin condensation and DNA degradation [66].

The extrinsic (death receptor-mediated) pathway is initiated by extracellular molecules [67]. Triggering of cell surface death receptors, characterized by members of the TNF receptor (TNFR) superfamily like TNFR-1 and Fas, evokes formation of a death-inducing signaling complex (DISC) [68]. The DISC brings procaspase-8 (or 10) molecules in close proximity to each other which results in their autoproteolytic activation [69]. Subsequently, active caspase-8 (or 10) is released into the cytosol and will cleave important executioner caspases like 3, 6 and 7. Caspase-8 is also involved in an indirect pathway linking extrinsic and intrinsic routes of apoptotic cell death [70,71]. In this pathway, activation of caspase-8 leads to the cleavage of Bid, a member of the B-cell lymphoma (Bcl)-2 family which induces permeability of the mitochondrial membrane. An important mediator of both the intrinsic and extrinsic apoptotic pathway is p53, a tumor suppressor molecule [72]. P53 is a transcription factor for several Bcl-2 family genes, including Bid and Bax, thereby promoting cytochrome *c* release from mitochondria and initiating the intrinsic apoptotic pathway. In addition, p53 induces APAF-1 expression which is required for formation of the apoptosome complex. P53 may also initiate the extrinsic apoptotic pathway through induction of Fas and activation of caspase-8.

Previously, *in vitro* studies demonstrated that mechanical stretch may activate apoptotic cell death [73-75]. Moreover, several investigators reported enhanced apoptosis after 2 to 5 hours of mechanical ventilation with high tidal volumes [76,77]. The importance of apoptotic cell death has been shown in experimental models of ALI/ARDS as well. Data by Fujita et al. indicate that immunogenic components of the outer membrane of gram-negative bacteria, lipopolysaccharides (LPS), may provoke apoptosis in a variety of pulmonary cells including vascular endothelial cells, bronchial and alveolar epithelial cells [78]. Since enhanced Fas/FasL signaling in ALI has been described in multiple studies [79-81], cell death via the extrinsic apoptotic pathway appears to play an essential role in the development of cellular injury during ALI/ARDS [82].

Necrotic cell death

It has been proposed that necrosis might provide a backup suicide mechanism when the caspase-dependent pathways cannot be properly activated [83,84], although this hypothesis may be too simplistic. Depletion of ATP for instance may favour a switch from apoptosis to

necrosis, in part because ATP is necessary for optimal activation of caspases [85]. Necrotic cell death is characterized by irreversible plasma membrane damage, cytoplasmic swelling, organelle breakdown and ultimately cell rupture with leakage of cellular contents into the extracellular space [84,86]. Consequently, this type of cell death is associated with a marked inflammatory response.

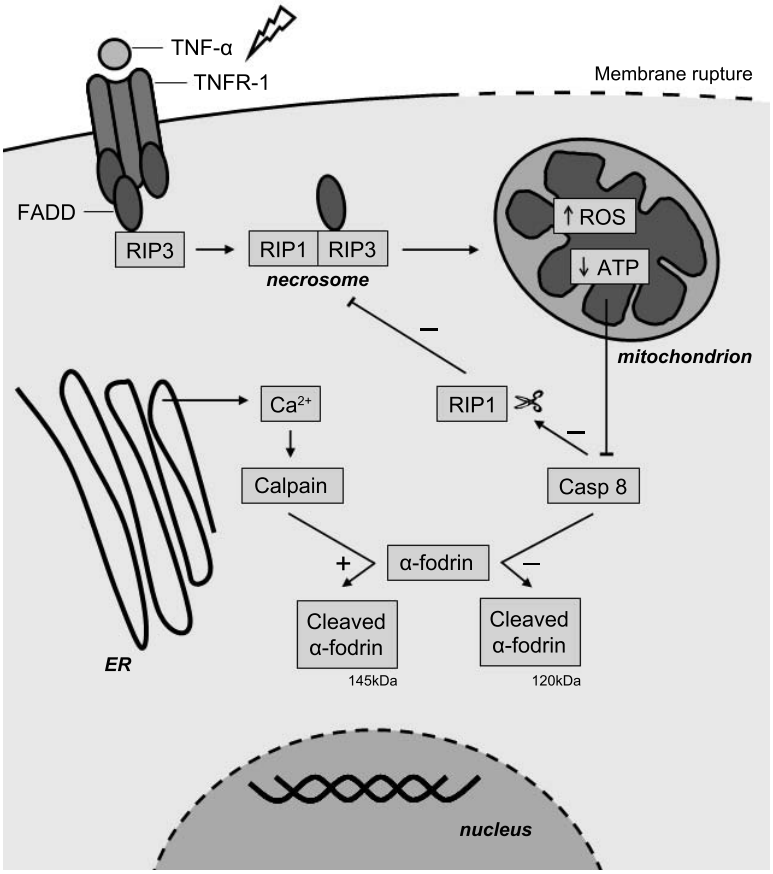

Figure 3: Pathways of necrotic cell death.
Triggering of TNFR-1 by TNF-α may also lead to programmed necrosis involving receptor-interacting protein (RIP) kinases. RIP3 has been implicated to act as an essential switch between TNF-induced apoptosis and necrosis. The protein complex containing RIP1/RIP3 consequently may function as a "necrosome" thereby promoting dysfunction of mitochondria, production of reactive oxygen species (ROS) and ultimately necrotic cell death. Depletion of adenosine triphosphate (ATP) may favour a switch from apoptosis to necrosis as well, partially because ATP is necessary for optimal activation of caspases. Moreover, necrotic cell death is associated with sustained elevation of cytosolic calcium (Ca^{2+}) levels. Ca^{2+} overload will activate calcium-dependent cysteine proteases (calpains) leading to cleavage of cytoskeletal components like spectrin (also known as fodrin) and disruption of organelle and cell integrity.
Casp = caspase (cysteine aspartyl-specific protease); FADD = Fas-associated protein with death domain; TNF = tumor necrosis factor; TNFR = TNF receptor.

For many years, necrosis has been considered to be an uncontrolled process. However, recent evidence suggests that the course of necrotic cell death might be as tightly regulated as apoptotic cell death (figure 3) [87,88]. It has to be kept in mind though, that necrotic and apoptotic traits might co-exist [89] and that the same cell death inducers may promote either necrosis or apoptosis depending on the specific environmental setting [90]. In this regard, previous research demonstrated that triggering of TNFR-1 by TNF-α does not only lead to activation of the extrinsic pathway of apoptosis but also to programmed necrosis involving receptor-interacting protein (RIP) kinases [90,91]. Data from Cho et al., He et al. and Zhang et al. implicate RIP3 as a pivotal switch between TNF-induced apoptosis and necrosis [92-94]. Based on this notion, the protein complex containing RIP1/RIP3 has been proposed to function as a "necrosome" [95]. In this setting, RIP3 will promote mitochondrial dysfunction, subsequent production of reactive oxygen species (ROS) and eventually necrotic cell death. Besides the production of ROS, necrotic cell death is associated with sustained elevation of cytosolic calcium (Ca^{2+}) levels [96]. Ca^{2+} overload leads to activation of calcium-dependent cysteine proteases, calpains. Active calpains promote release of lysosomal catabolic aspartyl proteases, cathepsins, which cleave essential cytoskeletal components like spectrin (also known as fodrin), microtubule subunits and microtubule-associated proteins thereby resulting in enhanced destabilization of the cell and ultimately in necrotic cell death [97,98].

Although the role of necrotic cell death has not been elucidated in VILI yet, it has been reported that mechanical stretch as such induces necrosis [75,99]. Furthermore, *in vitro* studies showed that LPS enhanced death of human pulmonary epithelial cells in a caspase-independent matter [100] suggesting that necrotic cell death pathways might be activated in lung diseases related to VILI, like ALI/ARDS.

Loss of vascular integrity

Under normal conditions, quiescent vascular endothelium establishes a tight barrier which controls the movement of plasma and/or leukocytes from the blood circulation into underlying tissue [101]. To achieve this barrier function, tight cell-cell contacts are formed by junctional transmembrane proteins such as vascular endothelial (VE)-cadherin [102].

One of the crucial systems regulating vascular cell integrity is the angiopoietin (Ang)-Tie2 system [103]. Ang-1 and Ang-2 are, respectively, an agonist and antagonist of the tyrosine kinase receptor Tie2 [104]. Constitutive Ang-1 expression and Tie2 phosphorylation in adult vasculature implies that Ang-1–mediated Tie2 signaling is required to maintain endothelial cell integrity and quiescence (figure 4) [103]. Binding of Ang-1 leads to autophosphorylation of Tie2 and subsequently activates several intracellular signaling pathways. One of the signal transduction routes that may be initiated is the phosphatidylinositol 3 kinase (PI3K)–Akt pathway which in general promotes cell survival [105-107]. In this regard, it has been shown that Ang-1–mediated Tie2 signaling may prevent endothelial cell apoptosis by activating Akt and by upregulating survivin, an apoptosis inhibitor [106]. Daly et al. proposed a molecular

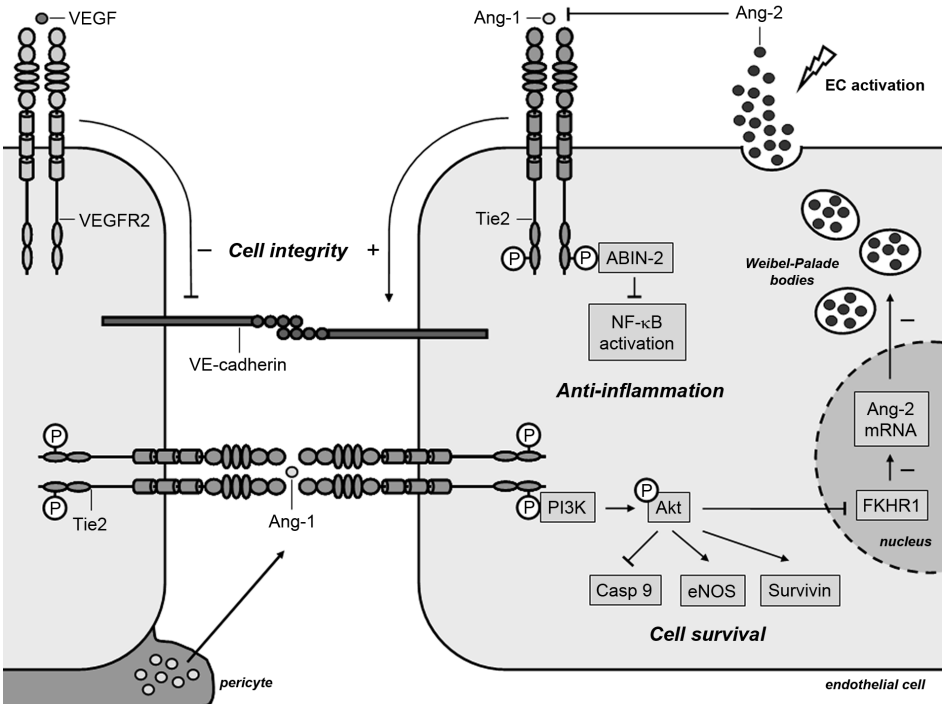

Figure 4: The angiopoietin (Ang)-Tie2 system.
Ang-1 and Ang-2 are, respectively, an agonist and antagonist of the tyrosine kinase receptor Tie2. Binding of Ang-1 leads to phosphorylation of Tie2 and subsequent activation of several intracellular signal transduction routes. *Cell survival:* Ang-1–mediated Tie2 phosphorylation activates the phosphatidylinositol 3 kinase (PI3K)–Akt pathway. Akt phosphorylation, in turn, will result in the negative regulation of caspase-9 and positive regulation of eNOS and survivin thereby preventing endothelial cell apoptosis. In addition, Akt phosphorylation may inactivate the forkhead transcription factor FKHR1 (FOXO1) which regulates genes associated with vascular destabilization (Ang-2). *Anti-inflammation:* Phosphorylated Tie2 interacts with A20-binding inhibitor of NF-κB (ABIN)-2, a NF-κB regulatory protein. Subsequently, Ang-1–mediated Tie2 phosphorylation will inhibit activation of the NF-κB pathway and lead to anti-inflammation and quiescence. *Cell integrity:* Ang-1–mediated Tie2 phosphorylation protects against vascular endothelial growth factor (VEGF)-stimulated permeability by inhibiting the VEGF-triggered endocytosis of vascular endothelial (VE)-cadherin. *In situations of endothelial activation:* Ang-2 proteins are released from Weibel-Palade bodies and compete with Ang-1 for binding to the Tie2 receptor, consequently altering vascular integrity, destabilizing the endothelial barrier and priming the endothelium to attain responsiveness to pro-inflammatory mediators.
Casp = caspase (cysteine aspartyl-specific protease); EC = endothelial cell; eNOS = endothelial nitric oxide synthase; NF-κB = nuclear factor-kappa B; P = phosphorylated; VEGFR2 = VEGF receptor 2.

mechanism through which Ang-1–mediated Akt activation may promote vascular stability [108]. These authors demonstrated that Akt activation inactivates the forkhead transcription factor FKHR1 (FOXO1) which regulates genes associated with endothelial cell apoptosis (survivin) and vascular destabilization (Ang-2). These findings imply that Ang-1–mediated

Tie2 signaling prevents Ang-2 expression and subsequent loss of vascular integrity [108]. Another signal transduction route that may be influenced upon Tie2 signaling is the nuclear factor-kappa B (NF-κB) pathway. Hughes et al. showed that Tie2 interacts with A20-binding inhibitor of NF-κB (ABIN)-2, a NF-κB regulatory protein [109]. As a consequence of ABIN-2 recruitment, Ang-1–mediated Tie2 signaling inhibits activation of the NF-κB pathway and results in an anti-inflammatory and quiescent state of the endothelial barrier. Finally, Gavard et al. described that Ang-1–mediated Tie2 signaling may protect against vascular endothelial growth factor (VEGF)-stimulated endothelial permeability by inhibiting the VEGF-triggered endocytosis of VE-cadherin [110].

Vascular leakage is an important feature of inflammatory disorders and is predominately caused by hyperpermeability of the endothelial barrier [111,112]. Until now, an increase in Ang-2 levels has been recognized to be the dynamic factor of the Ang-Tie2 system. In situations of endothelial activation, Ang-2 proteins are released from endothelial specific storage granules (Weibel-Palade bodies) and compete with Ang-1 for binding to the Tie2 receptor [103]. Consequently, Ang-2 may exert antagonistic functions on Ang-1–mediated Tie2 signaling and alter vascular integrity, destabilize the endothelial barrier and prime the endothelium to attain responsiveness to pro-inflammatory mediators [103]. In line with this, van der Heijden et al. demonstrated that circulating Ang-2 and increased Ang-2/Ang-1 ratios are related to pulmonary permeability edema and severity of ALI/ARDS in ventilated patients with or without sepsis [113]. Furthermore, Gallagher et al. showed that high circulating Ang-2 levels in ALI/ARDS patients are associated with a poor outcome [114]. Since 67% of these ALI/ARDS patients were mechanically ventilated, the authors proposed that ventilatory support alone does not cause enhanced Ang-2 levels. Considering abovementioned findings, therapeutic interventions that restore Ang-1–mediated Tie2 signaling might be beneficial for the clinical outcome of critically ill patients diagnosed with VILI or ALI/ARDS.

PREVENTING OR ATTENUATING VILI

The recognition that conventional mechanical ventilation may provoke detrimental effects has resulted in the introduction of "lung-protective" ventilation strategies [115]. Over the years, two important adjustments have been made. First, peak pressures and tidal volumes have been reduced to diminish the degree of alveolar stretching. Second, positive end-expiratory pressure (PEEP) has been implemented to avoid the cyclic opening and collapse of alveoli. In both experimental and clinical studies, the emergence of "lung-protective" ventilation strategies resulted in diminished ventilation-associated complications [15,116-118]. In this regard, the ARDS Network observed a 22% relative risk reduction in mortality rate when ventilating ALI/ARDS patients with "lung-protective" strategies [116]. Although adaptation of ventilation strategies has greatly improved the prognosis of the critically ill patient in the

ICU, the criteria how to optimally ventilate and oxygenate these patients remain a topic of debate.

Recent research provided striking evidence that even "lung-protective" mechanical ventilation may evoke VILI [30-32]. In this respect, adjustment of ventilator settings may not be sufficient to prevent an inflammatory reaction and/or injury in the lung and other organs. Additional therapeutic interventions are therefore urgently needed to protect critically ill patients against the detrimental effects caused by mechanical ventilation.

Additional therapeutic interventions

The awareness that macrophages, granulocytes and inflammatory mediators contribute to the development of lung diseases related to VILI, like ALI/ARDS, has resulted in the use of anti-inflammatory agents [119,120]. Glucocorticoids are a class of steroid hormones that exert their anti-inflammatory and immunosuppressive effects by binding to intracellular glucocorticoid receptors (GRs). After binding, the GR complex migrates from the cytosol to the nucleus where it regulates a host of gene activity, including inhibition of NF-κB and activator protein (AP)-1 driven expression of inflammatory genes [121]. Furthermore, glucocorticoids are known to suppress granulocyte recruitment and activation, preserve endothelial cell integrity and control vascular permeability [122,123]. The efficacy of glucocorticoids to treat ALI/ARDS in critically ill patients is still under debate [124-127]. In animal models of VILI, however, glucocorticoid therapy has shown promising results on downregulating lung inflammation and injury induced by mechanical ventilation. Ohta et al. observed attenuated lung dysfunction after methylprednisolone pre-treatment as indicated by a marked leftward shifting of the pressure-volume (P-V) curve in ventilated rats [122]. The authors proposed that the inhibitory effects of glucocorticoids on neutrophil activation and recruitment might have contributed to the improvement of lung function after mechanical ventilation. Supporting these results, other experimental studies demonstrated that dexamethasone administration diminishes ventilator-induced cytokine/chemokine release and consequently prevents lung injury [128,129]. Unfortunately, the anti-inflammatory effects of synthetic glucocorticoids cannot be separated from their metabolic effects. In particular when given at higher doses and for longer periods, systemic administration of glucocorticoids may lead to severe side effects such as hyperglycemia, deposition of body fat (weight gain), suppressed systemic immunity and increased susceptibility to infections [130]. One way to reduce the unwanted side effects of glucocorticoid treatment is to selectively deliver therapeutic agents into the diseased tissue [131]. Liposomes are valuable drug delivery systems as they may act as a depot from which the encapsulated drug will be slowly released to enable prolonged drug exposure at low concentrations [132]. Moreover, liposomes extravasate into tissues that experience increased capillary permeability [132] which facilitates their delivery at sites of inflammation or mechanical stretch. For specific drug delivery in inflamed tissue, endothelial

cells are attractive targets because of their important role in leukocyte transmigration and their direct contact with the blood stream. When modified with monoclonal antibodies, liposomal formulations may be selectively delivered to target epitopes of choice [133,134]. Inflammation-associated adhesion molecules, like E-selectin and VCAM-1, are appealing target epitope candidates due to their enhanced expression during inflammation and their prevalent endothelial location [131].

Taken together, the mechanisms underlying the pathogenesis of VILI and ALI/ARDS are very complex and may be modulated in several ways. So, pharmacological interventions that are designed to restore ventilator-induced endothelial activation, apoptotic/necrotic cell death and loss of vascular integrity might be advantageous in preventing chronic lung disease and improving clinical outcome in critically ill patients.

SYSTEMIC EFFECTS OF MECHANICAL VENTILATION

An important clinical observation is that most ventilated ALI/ARDS patients do not succumb to acute respiratory failure but rather to progressive non-pulmonary organ dysfunction, so-called multiple organ failure (MOF) [135-137]. A recent definition of MOF has been provided by John Marshall, who defined it as "the development of potentially reversible physiologic derangement involving two or more organ systems not involved in the disorder that resulted in ICU admission, and arising in the wake of a potentially life-threatening physiologic insult" [138]. In a clinical study, comprising 3147 patients from 198 ICUs in 24 European countries, Vincent et al. observed significant organ dysfunction in 71% of the ICU patients most of them being septic patients [139]. Moreover, they found a positive correlation between the number of organ systems failing and ICU mortality stressing the importance of MOF in the clinical outcome of critically ill patients. Supporting these clinical findings, data from experimental studies indicate that mechanical ventilation may cause detrimental effects in distal organs [129,140]. Imai et al. showed that mechanical ventilation with high tidal volumes induces epithelial cell apoptosis in the kidney and small intestine and causes elevation of serum markers implying renal dysfunction [140]. Moreover, ventilator-induced cardiovascular and hepatic injury has been reported as well [129].

Possible underlying mechanisms of MOF

Dysregulated immune responses appear to be essential for the development of MOF in critical illness [141]. In this regard, Roger Bone proposed five consecutive stages in the pathogenesis of MOF [142]. The first stage is initiated locally at the site of injury (local response). Pro-inflammatory mediators are released in the micro-environment to confine new damage and restore damage that already occurred [143,144]. To ensure that pro-inflammation does not

become destructive, anti-inflammatory mediators are produced. Homeostasis will be rapidly restored when the primary insult is limited and the patient is healthy. If the primary insult is more severe, however, pro-inflammatory mediators will be released into the systemic circulation thereby recruiting inflammatory cells to affected tissue (stage 2, systemic spill-over) [145,146]. The systemic release of anti-inflammatory mediators provides a compensatory mechanism that attenuates pro-inflammation and restores homeostasis under normal circumstances. In stage 3, regulation of the inflammatory response is lost and extensive release of pro-inflammatory mediators in the systemic environment ensues. Subsequent progression of endothelial dysfunction, enhancement of vascular permeability and obstruction of microvasculature by platelet sludging will lead to severe shock and comprised blood flow to vital organs (systemic inflammatory response syndrome, SIRS) [147-150]. This stage may be associated with elevated systemic levels of anti-inflammatory mediators which develops either *de novo* or as a response to the massive systemic pro-inflammation (stage 4, compensatory anti-inflammatory response syndrome, CARS [151]). The compensatory reaction may be as excessive as the pro-inflammatory response and may therefore result in pronounced immune suppression and augmented susceptibility to infections. If homeostasis cannot be re-established after stage 3 and 4, the final stage in the pathogenesis of MOF is reached (immunologic dissonance) [142]. Whether organ failure results from ongoing pro-inflammation of the systemic environment [152] and/or persistent suppression of the peripheral immune system [153], the dysbalance between pro- and anti-inflammation may ultimately cause increased mortality. This thesis will primarily focus on the inflammatory state of the systemic environment and its possible role in the development of ventilator-induced MOF.

Systemic inflammation

As a mechanism of ventilator-induced MOF, it has been suggested that lung inflammation may elicit release of inflammatory mediators into the circulation, thereby amplifying a pro-inflammatory systemic environment and eventually leading to detrimental effects in distal organs [43,154]. This concept was supported by clinical trials, where mechanical ventilation of ALI/ARDS patients did not only lead to an inflammatory response in pulmonary tissue but also enhanced cytokine levels in the circulation [38,155]. In animals with pre-existing lung injury, injurious mechanical ventilation increased permeability of the alveolar-capillary barrier and subsequently caused the release of inflammatory mediators into the systemic environment [156,157]. Pre-existing lung injury and implementation of injurious ventilation strategies, however, are not a requisite for the ventilator-induced release of inflammatory mediators as mechanical ventilation with clinically relevant settings may result in systemic inflammation as well [31].

Although there is quite some evidence that inflammatory mediators will be released from inflamed pulmonary tissue into the systemic circulation, a relationship between ventilator-induced spill-over and the pathogenesis of MOF has not been confirmed yet [158-160].

Therefore, it is tempting to speculate that other mechanisms may contribute to systemic inflammation as well. It has been thought that the physical stress of mechanical ventilation may activate the sympathetic nervous system [161]. Elenkov et al. and Straub et al. reported that sympathetic nerve terminals may evoke an inflammatory response in peripheral organs [162,163]. Local catecholamine release activates transcription factors, such as NF-κB, in leukocytes and macrophages thereby promoting IL-1β, TNF-α and IL-8 production, which in turn may result in an acute phase response in the liver [164,165]. Moreover, catecholamines are known to stimulate bacterial growth in the gastrointestinal system which may cause systemic inflammation through the translocation of enteric bacteria into the circulation [166].

OUTLINE OF THIS THESIS

An important finding from recent research is that "lung-protective" ventilation strategies with lower pressures or tidal volumes, which preserve alveolar integrity, may induce VILI as well [30-32]. Apart from improving current ventilation strategies, additional therapeutic interventions are urgently required to protect against the detrimental effects caused by mechanical ventilation. To design pharmacological therapies that may prevent ventilator-induced effects in the lung and distal organs, more insight is needed into the interaction between mechanical ventilation and local/systemic inflammatory mechanisms (**chapter 2**).

Dexamethasone, a widely used synthetic glucocorticoid, is known to successfully inhibit lung inflammation. However, systemic administration of glucocorticoids may induce severe side effects like increased blood glucose levels [167,168]. Local release of glucocorticoids by drug delivery systems such as liposomes could therefore be of therapeutic importance (**chapter 3**). It has been hypothesized that increased production of inflammatory mediators in the lung may precede lung injury during mechanical ventilation [39-41]. Therefore, we evaluated whether the anti-inflammatory effect of dexamethasone may protect against vascular leakage and impaired gas exchange induced by mechanical ventilation (**chapter 4**).

Loss of integrity of epithelial and endothelial monolayers has been recognized to be important in the ventilator-induced disruption of alveolar-capillary barriers [58]. Since Ang-1–mediated Tie2 signaling regulates vascular cell integrity [103], we investigated whether treatment with Ang-1 (a Tie2 receptor agonist) prevents ventilator-induced damage to alveolar-capillary barriers and subsequent increase in vascular leakage (**chapter 5**). Besides loss of cell integrity, apoptotic and/or necrotic cell death are thought to play a crucial role in the disruption of alveolar-capillary barriers [58]. Because enhanced permeability and edema formation are important characteristics of both VILI and ALI/ARDS, different cell death pathways were studied in a murine model of VILI and a rat model of LPS-induced ALI (**chapter 6**).

The main aims of this thesis are:

1. To investigate if ventilator-associated alveolar stretch may evoke endothelial activation and inflammation in healthy mice, not only in the lung but also in organs distal to the lung (**chapter 2**);
2. To study whether liposome-encapsulated dexamethasone may inhibit ventilator-induced lung inflammation and to evaluate whether targeting of Fcγ-receptors (FcγRs) on e.g. activated granulocytes and macrophages - by conjugating IgG to liposomes - may enhance the efficacy of dexamethasone-liposomes (**chapter 3**);
3. To determine if dexamethasone, a widely used anti-inflammatory synthetic glucocorticoid, may protect against vascular leakage and impaired gas exchange induced by mechanical ventilation (**chapter 4**);
4. To examine the effects of mechanical ventilation on the angiopoietin (Ang)-Tie2 system in lung tissue and to evaluate whether treatment with Ang-1 (Tie2 receptor agonist) may protect against inflammation, vascular leakage and impaired gas exchange induced by mechanical ventilation (**chapter 5**);
5. To investigate the relative contribution of caspase-dependent and caspase-independent signaling pathways in a rat model of LPS-induced ALI, a widely used model for acute respiratory failure (**chapter 6**).

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CHAPTER

2

Ventilator-induced endothelial activation and inflammation in the lung and distal organs

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ABSTRACT

Background: Results from clinical studies have provided evidence for the importance of leukocyte-endothelial interactions in the pathogenesis of pulmonary diseases such as acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), as well as in systemic events like sepsis and multiple organ failure (MOF). The present study was designed to investigate whether alveolar stretch due to mechanical ventilation may evoke endothelial activation and inflammation in healthy mice, not only in the lung but also in organs distal to the lung.

Methods: Healthy male C3H/HeN mice were anesthetized, tracheotomized and mechanically ventilated for either 1, 2 or 4 hours. To study the effects of alveolar stretch *in vivo*, we applied a ventilation strategy that causes overstretch of pulmonary tissue, i.e. 20 cmH_2O peak inspiratory pressure (PIP) and 0 cmH_20 positive end-expiratory pressure (PEEP). Non-ventilated, sham-operated animals served as a reference group (non-ventilated controls, NVC).

Results: Alveolar stretch imposed by mechanical ventilation did not only induce *de novo* synthesis of adhesion molecules in the lung but also in organs distal to the lung, like liver and kidney. No activation was observed in the brain. In addition, we demonstrated elevated cytokine and chemokine expression in pulmonary, hepatic and renal tissue after mechanical ventilation which was accompanied by enhanced recruitment of granulocytes to these organs.

Conclusions: Our data implicate that mechanical ventilation causes endothelial activation and inflammation in mice without pre-existing pulmonary injury, both in the lung and distal organs.

INTRODUCTION

Critically ill patients in the intensive care unit often require mechanical ventilation to adequately oxygenate vital organs. Although artificial ventilation is lifesaving, the procedure itself may lead to serious damage in both healthy and diseased lungs [1]. Studies have revealed that the cyclic opening and collapse of alveoli during mechanical ventilation may provoke alveolar stretch and subsequently result in ventilator-induced lung injury (VILI) [2,3]. Important features of VILI are increased cytokine and chemokine production, alveolar-capillary permeability, protein-rich edema formation and ultimately impaired gas exchange [4-6].

Pro-inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α are secreted by alveolar macrophages upon mechanical stretch [7] and are capable of stimulating endothelial activation [8]. In turn, cytokine-activated endothelial cells secrete chemokines and express adhesion molecules on their surface resulting in enhanced leukocyte adhesiveness and transmigration of activated immune cells across the endothelium of inflamed tissue [8-10]. Vascular adhesion molecules that belong to the selectin family (P-selectin and E-selectin) mediate leukocyte margination and rolling along the blood vessel wall, whereas members of the immunoglobulin (Ig) superfamily (vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1 and platelet-endothelial cell adhesion molecule (PECAM)-1) participate in leukocyte adhesion and transmigration into underlying tissue [11]. Previously, it has been shown that soluble adhesion molecule levels are elevated in patients with serious lung diseases such as acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) [12,13]. Moreover, augmented P-selectin, VCAM-1 and ICAM-1 expression was found in pulmonary tissue after mechanical ventilation [14] suggesting that adhesion molecules may play a crucial role in the pathogenesis of VILI.

Most critically ill patients do not succumb to lung deterioration associated with mechanical ventilation but to multiple organ failure (MOF) caused by a systemic inflammatory response syndrome [15,16]. As a mechanism of MOF, it has been hypothesized that ventilator-induced lung inflammation may elicit release of inflammatory mediators into the circulation, thereby amplifying a pro-inflammatory systemic environment and eventually leading to detrimental effects in distal organs [17-19]. As high levels of inflammatory mediators in the periphery are thought to be important in the pathogenesis of MOF [20,21], systemic effects of mechanical ventilation have been proposed to be responsible [18,19]. Similar to sepsis-induced MOF [22,23], activation of endothelial cells in distal organs might be essential in the development of ventilator-induced MOF.

We designed this study to investigate whether ventilator-induced alveolar stretch may cause endothelial activation in healthy mice, not only in the lung but also in organs distal to the lung. To determine endothelial activation in these organs we assessed *de novo* synthesis of adhesion molecules. Moreover, we examined ventilator-induced effects on the inflammatory state of pulmonary, hepatic, renal and cerebral tissue.

MATERIALS AND METHODS

Animals

The experiments were performed in accordance with international guidelines and approved by the experimental animal committee of the Erasmus Medical Center Rotterdam. A total of 42 adult male C3H/HeN mice (Harlan CPB, Zeist, the Netherlands), weighing 25 to 30 grams, were randomly assigned to different experimental groups.

Study design

To investigate the effects of alveolar stretch *in vivo*, we applied a mechanical ventilation strategy that has been described to cause overstretch of pulmonary tissue [24,25]. The method of mechanical ventilation was based on the first experiments performed in mice [26]. Thirty mice were tracheotomized under inhalation anesthesia (65% nitrous oxide, 33% oxygen, 2% isoflurane; Pharmachemie, Haarlem, the Netherlands). Subsequently, anesthesia was continued with 24 mg/kg/h intraperitoneal sodium pentobarbital (Algin, Maassluis, the Netherlands). Additional anesthesia was given when necessary. The intraperitoneal administered anesthesia fluid was sufficient to correct for hypovolemia. Muscle relaxation was attained with 0.4 mg/kg/h intramuscular pancuronium bromide (Organon Technika, Boxtel, the Netherlands). The animals were connected to a Servo Ventilator 300 (Siemens-Elema, Solna, Sweden) and ventilated for 1, 2 or 4 hours in a pressure-controlled time-cycled mode (n = 9 to 10 per group), at a fractional inspired oxygen concentration (FiO_2) of 1.0, inspiration-to-expiration ratio of 1:2 and frequency of 20 to 30 breaths/min to maintain normocapnia. Peak inspiratory pressure (PIP) was set at 20 cmH_2O and positive end-expiratory pressure (PEEP) at 0 cmH_2O. A polyethylene catheter was inserted into the carotid artery and blood gas determinations were performed using a pH/blood gas analyzer (ABL 505; Radiometer, Copenhagen, Denmark). Body temperature was maintained between 36 and 38°C with a heating device (UNO Roestvaststaal, Zevenaar, the Netherlands). Eight healthy non-ventilated, sham-operated mice served as a reference group (non-ventilated controls, NVC). To investigate whether the high partial pressure of arterial oxygen (PaO_2) levels associated with our ventilation strategy may contribute to changes in the immune response, spontaneously breathing animals (n = 6) were placed in an oxygen saturated box for 4 hours (FiO_2 of 1.0, hyperoxia). This exposure time was chosen, because it resembles the longest period of time that mice were subjected to mechanical ventilation. All animals were sacrificed with an overdose of intraperitoneal sodium pentobarbital (Organon, Oss, the Netherlands).

Histopathology

Two mice per group were perfused with PBS. Pulmonary tissue was directly removed, frozen in liquid nitrogen and stored at -80°C to evaluate lung architecture and presence of granulocytes. Before being snap frozen, lungs were filled with Tissue-Tek® (Sakura Finetek, Zoeterwoude, the Netherlands).

Cryosections (5 µm) were cut on a cryostat microtome (Leica Microsystems, Nussloch, Germany) and fixed with acetone for 10 minutes. To assess pulmonary histopathology, longitudinal sections were stained with hematoxylin-eosin (H&E; Klinipath, Duiven, the Netherlands).

Tissue homogenate preparation

Pulmonary, hepatic, renal and cerebral tissue (from 4 to 8 mice per group) was directly removed and frozen in liquid nitrogen to evaluate endothelial activation and inflammation. Tissues were pulverized using a liquid nitrogen-cooled mortar and pestle, and divided in several fractions allowing us to use the lung, liver, kidney and brain from one animal for multiple analyses (as described below).

Real-time RT-PCR analysis

Total RNA was isolated from pulverized tissues with TRIzol® reagent (Invitrogen, Paisley, UK). cDNA was synthesized from total RNA with SuperScript Reverse Transcriptase (Invitrogen). PCR reaction was performed with iQ5 Real-Time PCR Detection System (Biorad, Hercules, CA) using primers for E-selectin, VCAM-1, ICAM-1, PECAM-1, IL-1β, TNF-α

Table 1. Primers used for real-time RT-PCR

	Forward	Reverse
E-selectin	CAACgTCTAggTTCAAAACAATCAg	TTAAgCAggCAAgAggAACCA
VCAM-1	TgAAgTTggCTCACAATTAAgAAgTT	TgCgCAgTAgAgTgCAAggA
ICAM-1	ggAgACgCAgAggACCTTAACAg	CgACgCCgCTCAgAAgAACC
PECAM-1	ACgATgCgATggTgTATAAC	ACCTTgggCTTggATACg
IL-1β	CAACCAACAAgTgATATTCTCCATg	gATCCACACTCTCCAgCTgCA
TNF-α	gCggTgCCTATgTCTCAg	gCCATTTgggAACTTCTCATC
KC	AAAAggTgTCCCCAAgTAACg	gTCAgAAgCCAgCgTTCAC
β-actin	AgAgggAAATCgTgCgTgAC	CAATAgTgATgACCTggCCgT
GAPdH	TgAAgCAggCATCTgAggg	CgAAggTggAAgAgTgggAg

GAPdH = glyceraldehyde 3-phosphate dehydrogenase; ICAM = intercellular adhesion molecule; IL = interleukin; KC = keratinocyte-derived chemokine; PECAM = platelet-endothelial cell adhesion molecule; TNF = tumor necrosis factor; VCAM = vascular cell adhesion molecule.

and keratinocyte-derived chemokine (KC; murine homologue of IL-8; table 1). To confirm appropriate amplification, size of PCR products was verified by agarose gel separation. Data were normalized for expression of internal controls, i.e. the average value of β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPdH).

Myeloperoxidase (MPO) assay

For lung and brain, MPO activity was determined as described previously [27]. In short, pulverized tissues were homogenized in 50 mM HEPES buffer (pH 8.0), centrifuged and pellets were homogenized again in water and 0.5% cetyltrimethylammonium chloride (CTAC; Merck, Darmstadt, Germany). After centrifugation, supernatants were diluted in 10 mM citrate buffer (pH 5.0) and 0.22% CTAC. Substrate solution containing 3 mM 3',5,5'-tetramethylbenzidine dihydrochloride (TMB; Sigma-Aldrich, Steinheim, Germany), 120 μM resorcinol (Merck) and 2.2 mM hydrogen peroxide (H_2O_2) in distilled water was added. Reaction mixtures were incubated for 20 minutes at room temperature and stopped by addition of 4M sulfuric acid (H_2SO_4) followed by determination of optical density at 450 nm. MPO activity of a known amount of MPO units (Sigma-Aldrich) was used as reference. For liver and kidney, pulverized tissues were homogenized in lysis buffer with protease inhibitors. In supernatants, MPO activity was analyzed by ELISA according to the manufacturer's instructions (Hycult Biotechnology, Uden, the Netherlands). To correct for homogenization procedures, total protein concentration of samples was determined with a BCA protein-assay (Pierce Biotechnology, Rockford, IL) using BSA as standard.

Statistical analysis

Data are expressed as mean ± SEM. All parameters were analyzed by one-way ANOVA with least significant difference (LSD) post-hoc test. P-values less than 0.05 were considered statistically significant.

RESULTS

Stability of the model

Mechanical ventilation was applied to healthy mice to induce alveolar stretch. All mice survived the ventilatory protocol and produced urine throughout the experiment. Arterial blood gas analysis of ventilated mice showed a stable oxygen tension (PaO_2) with carbon dioxide tension ($PaCO_2$), pH and base excess (BE) within the physiological range (table 2). In addition, pulmonary architecture was preserved during the experiment (figure 1).

Table 2. Oxygenation variables after 1, 2 and 4 hours of mechanical ventilation

Time (hours)	PaO_2	$PaCO_2$	pH	BE
1	568.6 ± 23.1	31.0 ± 2.8	7.46 ± 0.02	-0.8 ± 1.4
2	509.7 ± 22.0	35.4 ± 2.3	7.42 ± 0.02	-1.5 ± 0.6
4	493.4 ± 24.9	45.4 ± 4.3	7.32 ± 0.03	-4.0 ± 0.9

Data are expressed as mean ± SEM. PaO_2 = partial pressure of arterial oxygen in mmHg; $PaCO_2$ = partial pressure of arterial carbon dioxide in mmHg; BE = base excess in mmol/l.

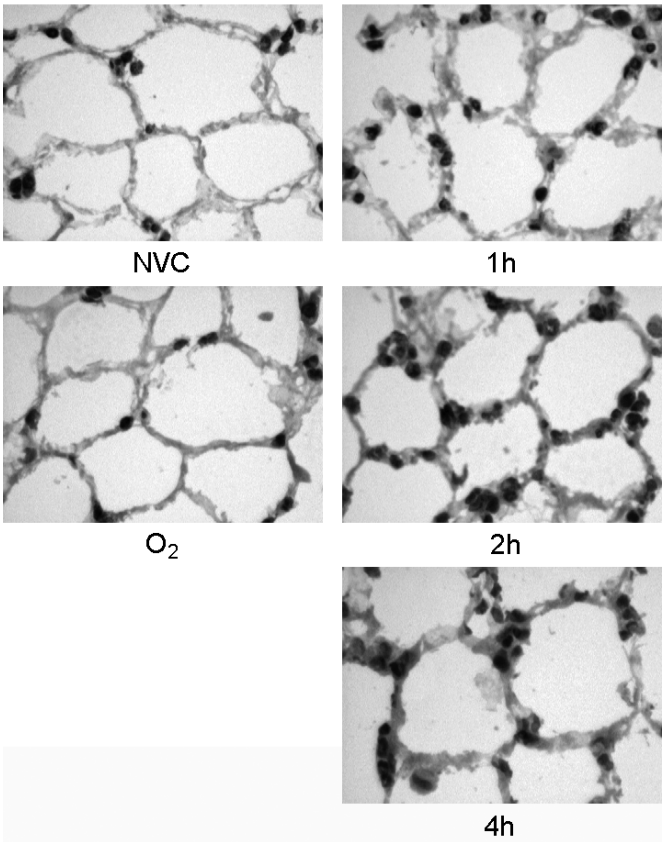
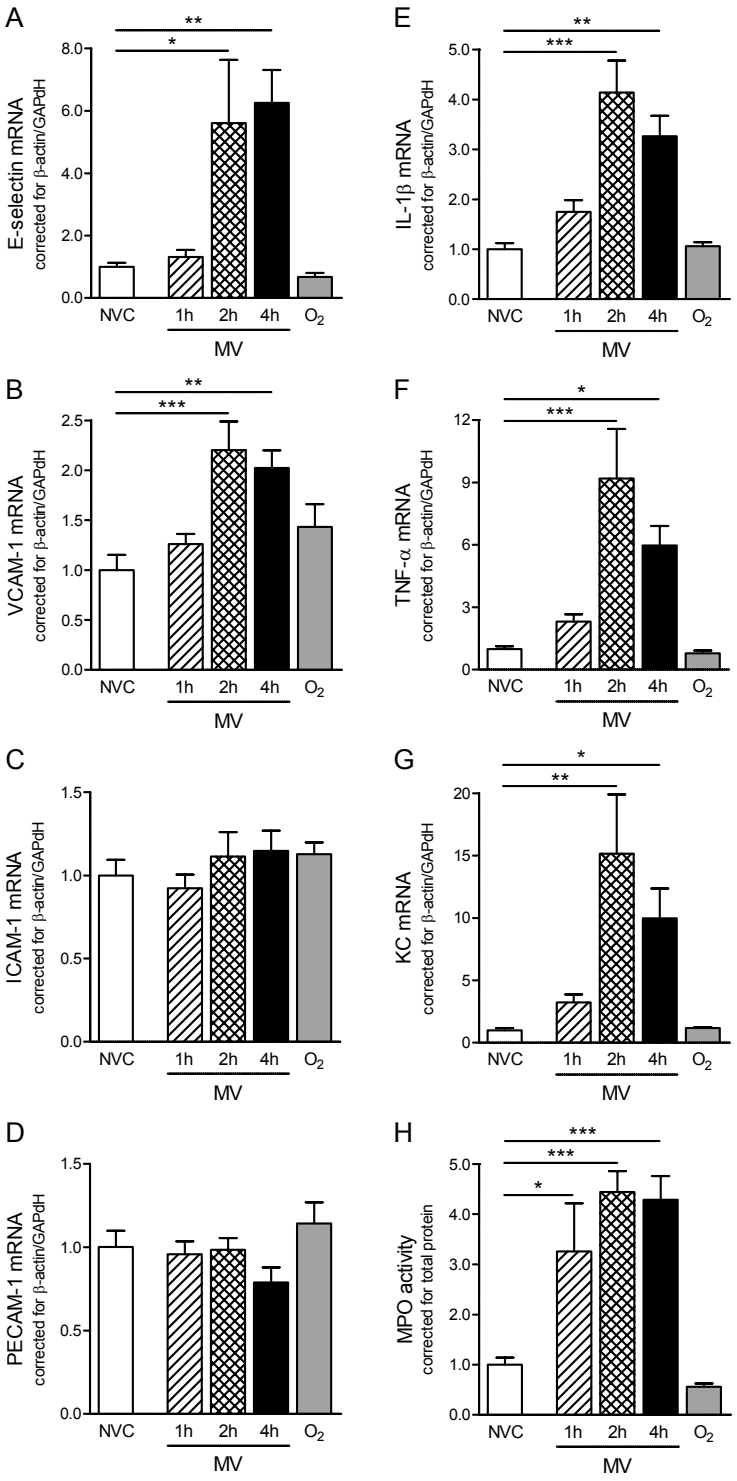


Figure 1: Histopathology of pulmonary tissue. (See color figure on pg. 195.)
Lung sections were stained with hematoxylin-eosin (H&E) to analyze lung architecture and presence of granulocytes in pulmonary tissue. Magnification x500.
NVC = non-ventilated controls; 1h, 2h, 4h = mechanically ventilated for 1, 2 or 4 hours; O_2 = hyperoxia for 4 hours.


A
E-selectin mRNA
corrected for β-actin/GAPdH
B
VCAM-1 mRNA
corrected for β-actin/GAPdH
C
ICAM-1 mRNA
corrected for β-actin/GAPdH
D
PECAM-1 mRNA
corrected for β-actin/GAPdH
E
IL-1β mRNA
corrected for β-actin/GAPdH
F
TNF-α mRNA
corrected for β-actin/GAPdH
G
KC mRNA
corrected for β-actin/GAPdH
H
MPO activity
corrected for total protein
NVC
1h
2h
4h
O2
MV

Effects of mechanical ventilation on inflammatory state of pulmonary tissue

Endothelial activation

We studied the effect of mechanical ventilation on endothelial activation in pulmonary tissue by measuring *de novo* synthesis of adhesion molecules. Compared to NVC, enhanced mRNA expression of E-selectin and VCAM-1 was noticed after 2 and 4 hours of mechanical ventilation (figures 2a and b). No ventilator-induced changes in ICAM-1 and PECAM-1 mRNA were found in the lung (figures 2c and d).

Cytokine and chemokine expression

After 2 and 4 hours of mechanical ventilation, significantly higher mRNA expression of the pro-inflammatory cytokines IL-1β and TNF-α were observed (figures 2e and f). In addition, mechanical ventilation induced an increase in mRNA expression of the chemokine KC (figure 2g).

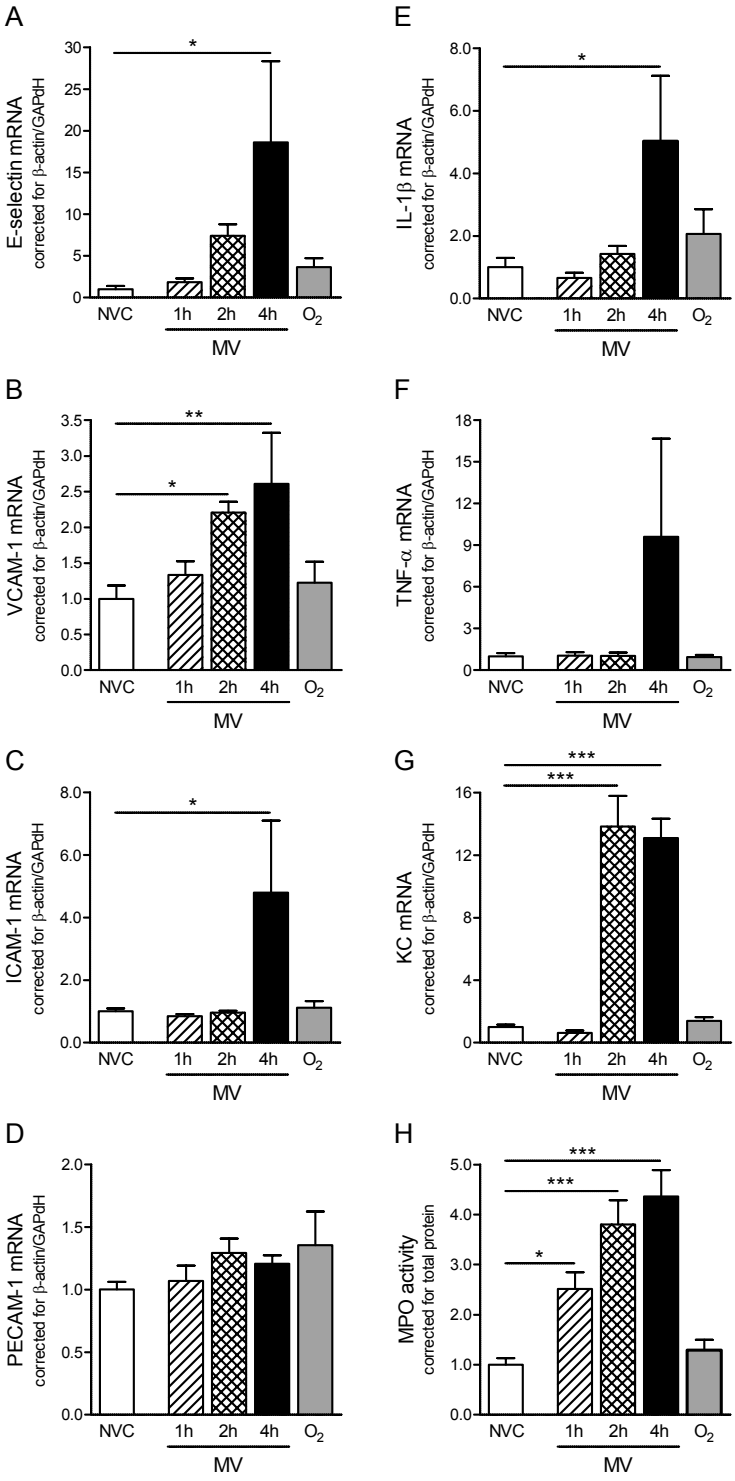
Granulocyte recruitment

To investigate whether the ventilator-induced endothelial activation and chemokine expression was accompanied by recruitment of granulocytes to inflamed pulmonary tissue, MPO activity was determined in total lung homogenates (figure 2h). Elevated MPO activity was found after 1, 2 and 4 hours of mechanical ventilation, which correlated with the presence of granulocytes observed in pulmonary sections stained for H&E (figure 1). Furthermore, histology of ventilated lungs showed margination of granulocytes to the blood vessel wall. Exudation of granulocytes into the alveolar space was not observed.

Contribution of hyperoxia

To examine whether the high PaO_2 levels associated with our ventilation strategy contributed to changes in the pulmonary immune response, we exposed spontaneously breathing mice to 100% oxygen levels for 4 hours (FiO_2 of 1.0, hyperoxia). As depicted in figures 1 and 2,

Figure 2: Ventilator-induced endothelial activation and inflammation in pulmonary tissue.
A-D: In total lung homogenates, mRNA expression of the adhesion molecules E-selectin, vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1 and platelet-endothelial cell adhesion molecule (PECAM)-1 was determined by real-time RT-PCR. **E-G:** In addition, mRNA expression of the pro-inflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α and the chemokine keratinocyte-derived chemokine (KC) was determined. Levels were normalized for expression of internal controls, i.e. the average value of β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPdH). **H:** In total lung homogenates, myeloperoxidase (MPO) activity was determined as a measure of granulocyte infiltration. Levels were normalized for total protein levels. Data are expressed as mean ± SEM of 4 to 8 mice for each group, and depicted relative to NVC. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.
NVC = non-ventilated controls; 1h, 2h, 4h = mechanically ventilated for 1, 2 or 4 hours; O_2 = hyperoxia for 4 hours.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E
IL-1β mRNA
corrected for β-actin/GAPdH
F
TNF-α mRNA
corrected for β-actin/GAPdH
G
KC mRNA
corrected for β-actin/GAPdH
H
MPO activity
corrected for total protein
NVC
1h
2h
4h
O2
MV

hyperoxia-exposed mice (O_2 group) showed a similar adhesion molecule, cytokine/chemokine expression, MPO activity and lung histopathology compared to NVC.

Effects of mechanical ventilation on inflammatory state of hepatic, renal and cerebral tissue

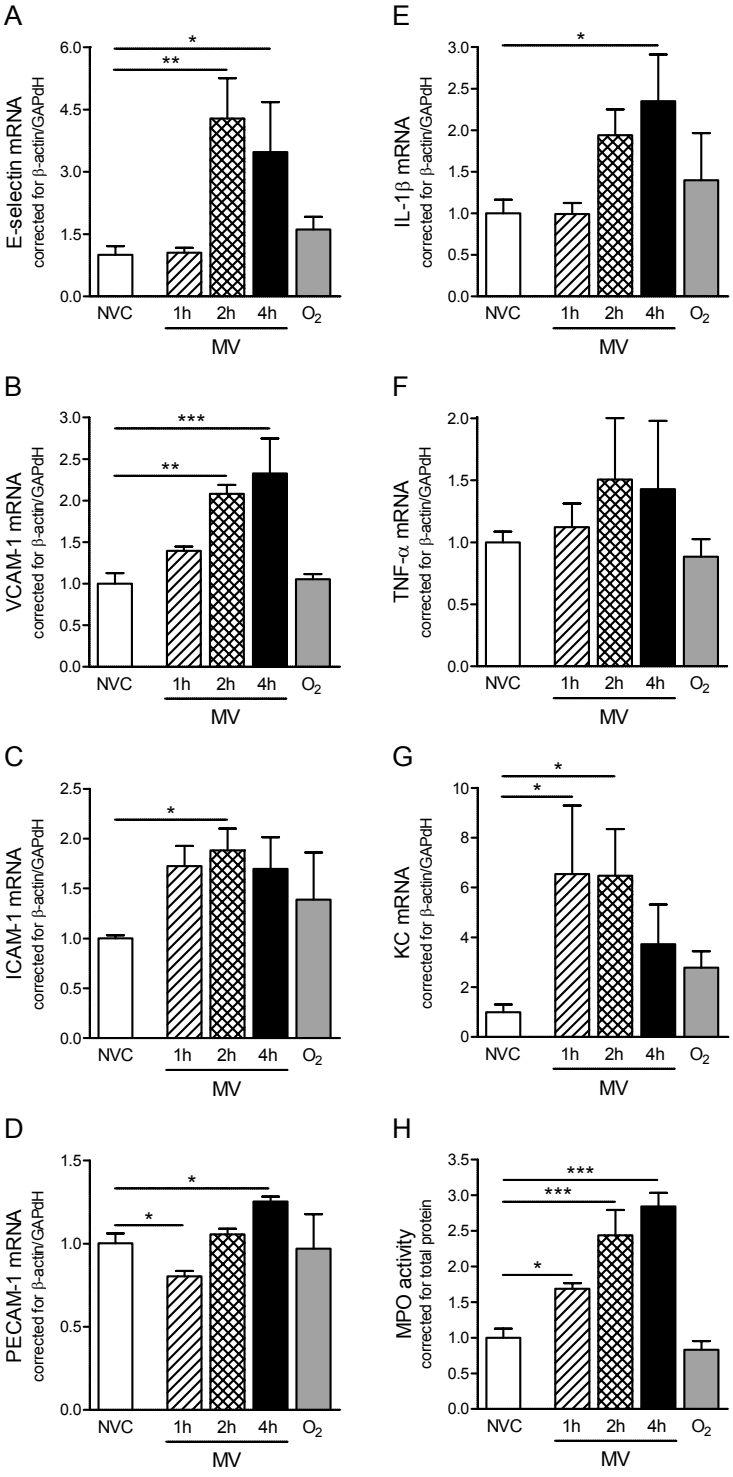
Endothelial activation

The effect of mechanical ventilation on endothelial activation in hepatic, renal and cerebral tissue was investigated by analyzing *de novo* synthesis of adhesion molecules. In the liver, higher mRNA expression of E-selectin and ICAM-1 was observed after 4 hours of mechanical ventilation in comparison with NVC (figures 3a and c). VCAM-1 mRNA was already elevated in hepatic tissue after 2 hours of mechanical ventilation and further increased after 4 hours (figure 3b). No differences were found in PECAM-1 mRNA (figure 3d). Also in the kidney, we noticed increased mRNA expression of E-selectin, VCAM-1 and ICAM-1 after 2 and 4 hours of mechanical ventilation (figures 4a to c). Minimal changes in PECAM-1 mRNA were found in renal tissue of ventilated mice (figure 4d). In the brain, mechanical ventilation did not induce a significant change in adhesion molecule mRNA expression as compared to NVC (data not shown).

Cytokine and chemokine expression

In the liver, IL-1β and TNF-α mRNA expression was enhanced after 4 hours of mechanical ventilation (figures 3e and f) although the difference in TNF-α mRNA between 4 hours of mechanical ventilation and NVC did not reach statistical significance (p=0.09). KC mRNA was significantly elevated in hepatic tissue after 2 hours of mechanical ventilation and further increased after 4 hours (figure 3g). In the kidney of ventilated mice, we noticed higher IL-1β mRNA expression at four hours whereas no changes were found in TNF-α mRNA expression (figures 4e and f). Increased KC mRNA was already present after 1 and 2 hours of ventilation (figure 4g). In the brain, mechanical ventilation did not induce a detectable cytokine or chemokine response (data not shown).

Figure 3: Ventilator-induced endothelial activation and inflammation in hepatic tissue.
A-D: In total liver homogenates, mRNA expression of the adhesion molecules E-selectin, vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1 and platelet-endothelial cell adhesion molecule (PECAM)-1 was determined by real-time RT-PCR. **E-F:** In addition, mRNA expression of the pro-inflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α and the chemokine keratinocyte-derived chemokine (KC) was determined. Levels were normalized for expression of internal controls, i.e. the average value of β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPdH). **H:** In total liver homogenates, myeloperoxidase (MPO) activity was determined as a measure of granulocyte infiltration. Levels were normalized for total protein levels. Data are expressed as mean ± SEM of 4 to 8 mice for each group, and depicted relative to NVC. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.
NVC = non-ventilated controls; 1h, 2h, 4h = ventilated for 1, 2 or 4 hours; O_2 = hyperoxia for 4 hours.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corrected for β-actin/GAPdH
E
IL-1β mRNA
corrected for β-actin/GAPdH
F
TNF-α mRNA
corrected for β-actin/GAPdH
G
KC mRNA
corrected for β-actin/GAPdH
H
MPO activity
corrected for total protein
NVC
1h
2h
4h
O2
MV

Figure 4: Ventilator-induced endothelial activation and inflammation in renal tissue.
A-D: In total kidney homogenates, mRNA expression of the adhesion molecules E-selectin, vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1 and platelet-endothelial cell adhesion molecule (PECAM)-1 was determined by real-time RT-PCR. E-F: In addition, mRNA expression of the pro-inflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α and the chemokine keratinocyte-derived chemokine (KC) was determined. Levels were normalized for expression of internal controls, i.e. the average value of β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPdH). **H:** In total kidney homogenates, myeloperoxidase (MPO) activity was determined as a measure of granulocyte infiltration. Levels were normalized for total protein levels. Data are expressed as mean ± SEM of 4 to 8 mice for each group, and depicted relative to NVC. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. NVC = non-ventilated controls; 1h, 2h, 4h = mechanically ventilated for 1, 2 or 4 hours; O_2 = hyperoxia for 4 hours.

Granulocyte recruitment

To determine whether enhanced endothelial activation and chemokine expression was accompanied by recruitment of granulocytes to inflamed distal organs, we analyzed MPO activity in hepatic, renal and cerebral tissue. In the liver of ventilated mice, enhanced MPO activity was observed already at 1 hour and was most pronounced at 4 hours (figure 3h). Also in renal tissue, MPO activity was higher after 1 hour of mechanical ventilation and increased further at 2 and 4 hours (figure 4h). In the brain, MPO activity was below detection level in all experimental groups (data not shown).

Contribution of hyperoxia

We examined if the high oxygen levels associated with our ventilation strategy might contribute to changes in the immune response of distal organs by exposing spontaneously breathing mice to 100% oxygen levels for 4 hours. Figures 3 and 4 illustrate that *de novo* synthesis of adhesion molecules, cytokines/chemokines and MPO activity were comparable in hepatic and renal tissue of hyperoxia-exposed mice and NVC.

DISCUSSION

To investigate the effects of alveolar stretch on endothelial activation and inflammation in the lung and distal organs, healthy mice were exposed to a ventilation strategy that has been described to cause overstretch of pulmonary tissue [24,25]. During 4 hours of mechanical ventilation, blood gas values remained within the physiological range and pulmonary architecture was preserved suggesting that the cardio-pulmonary integrity was maintained throughout the experiment. Our major finding was that mechanical ventilation induced *de novo* synthesis of various adhesion molecules represented by an elevation of E-selectin and VCAM-1 mRNA in pulmonary tissue and a rise in E-selectin, VCAM-1 and ICAM-1 mRNA in hepatic and renal tissues but not in cerebral tissue. Moreover, we noticed a time-dependent

increase in cytokine and chemokine mRNA expression after mechanical ventilation which was accompanied by elevated recruitment of granulocytes. Importantly, this enhanced pro-inflammatory state was found both in the lung and organs distal to the lung.

There is convincing evidence that leukocyte-endothelial interactions play a crucial role in the pathogenesis of serious inflammatory diseases related to VILI, such as ALI and ARDS [28,29]. Gando et al. observed that soluble levels of P-selectin, E-selectin, ICAM-1 and VCAM-1 were enhanced within 24 hours after the diagnosis of ALI/ARDS [13]. Furthermore, these authors showed a marked increase in these soluble adhesion molecules when subdividing patients into survivors and non-survivors implying that adhesion molecules may have prognostic value for the development and clinical outcome of ALI/ARDS. The present study demonstrates that alveolar stretch imposed by mechanical ventilation induces activation of pulmonary endothelium in healthy mice, as measured by higher mRNA expression of E-selectin and VCAM-1. Our results are supported by *in vitro* models of cyclic strain and shear stress showing increased endothelial expression of adhesion molecules from the selectin family and Ig superfamily [30,31]. Therefore, it appears that ventilator-induced endothelial activation facilitates migration and adhesiveness of activated immune cells to inflamed pulmonary tissue, which in turn may lead to tissue injury. Although mechanical ventilation enhanced the number of granulocytes and expression of pro-inflammatory cytokines/chemokines in the lung, significant changes in pulmonary architecture and oxygenation variables were not observed. In line with this, recent studies demonstrated that ventilation strategies that do not cause deterioration of pulmonary function *per se* are capable of provoking ventilator-induced lung inflammation [25,32].

To our knowledge, only one other study investigated the effect of mechanical ventilation on expression of cell-bound adhesion molecules. Miyao et al. described that ventilation with high tidal volumes enhances P-selectin, VCAM-1 and ICAM-1 expression in pulmonary vasculature of healthy rats [14]. We observed that mechanical ventilation did not only cause upregulation of adhesion molecules in the lung but also evoked *de novo* synthesis of E-selectin, VCAM-1 and ICAM-1 in organs distal to the lung, such as liver and kidney. Whether a dose-response relation exists between the extent of alveolar stretch and effects on distal organs remains to be determined. It has been proposed previously that an elevation of adhesion molecule expression might contribute to tissue injury and ultimately to MOF by facilitating leukocyte activation and migration [22,23]. In line with this notion, we demonstrated that mechanical ventilation augments KC mRNA expression and MPO activity in hepatic and renal tissue. Our data indicate that alveolar stretch due to mechanical ventilation promotes endothelial activation, inflammatory mediator production and the presence of granulocytes in distal organs. Therefore, we propose that mechanical ventilation may play a significant role in the pathogenesis of MOF. Combined with other events, such as an endotoxin challenge, ventilator-induced effects on the lung and distal organs will be

exacerbated [33,34] and possibly underlie the high incidence of MOF in critically ill patients ventilated with high pressures or tidal volumes [35].

Studies describing the combined effects of high PaO_2 levels and mechanical ventilation have revealed that hyperoxia may aggravate VILI [36,37]. Li et al. have shown augmented lung injury in mice exposed to mechanical ventilation with high tidal volumes and hyperoxia compared to animals ventilated with room air [36]. Therefore, we investigated whether the high PaO_2 levels associated with our mechanical ventilation strategy were contributing to the observed changes in inflammatory mediator expression and granulocyte recruitment. In our study, hyperoxia as such did not lead to pulmonary endothelial activation and inflammation. Furthermore, we noticed that the high PaO_2 levels did not induce an augmented immune response in organs distal to the lung. Although we cannot exclude that hyperoxia is aggravating the stretch-induced inflammatory response in pulmonary tissue, we consider that effects of high PaO_2 levels on the inflammatory state of the liver and kidney will not be the primary cause of distal organ activation. As the reference group in our study (NVC) could not be sedated for the same period as ventilated animals, we cannot exclude that anesthesia affects endothelial activation and inflammation by itself. However, we have previously shown that mechanical ventilation with injurious settings (PIP 32/PEEP 6) increased pulmonary macrophage inflammatory protein (MIP)-2 expression and reduced splenocyte natural killer cell activity, whereas mechanical ventilation with protective settings (PIP 14/PEEP 6) did not have these effects on inflammation [24]. Since the same type of anesthesia was applied in these two groups, we propose that anesthesia as such does not induce the inflammatory response.

Taken together, we demonstrated that mechanical ventilation induces endothelial activation and inflammation in the lung but also in the liver and kidney. It remains to be determined which factors lead to the onset of this inflammatory response in distal organs. Haitsma et al. and Tutor et al. have shown that ventilator-induced permeability of the alveolar-capillary barrier causes release of inflammatory mediators into the systemic circulation [38,39]. In the present study, alveolar stretch due to mechanical ventilation enhanced mRNA expression of adhesion molecules and cytokines/chemokines in hepatic and renal tissue, thus inducing *de novo* synthesis of these mediators in organs distal to the lung. Furthermore, we observed granulocyte recruitment to the liver and kidney, and KC mRNA expression in the kidney already after 1 hour of mechanical ventilation. These results indicate that ventilator-induced changes of the immune response may occur simultaneously in the lung, liver and kidney, suggesting that release of inflammatory mediators into the circulation is probably not the only cause of augmented endothelial activation and inflammation in distal organs. We cannot exclude, however, that cytokines in the systemic circulation induce *de novo* synthesis of adhesion molecules, cytokines and chemokines in distal organs.

It has been hypothesized that the physical stress of mechanical ventilation activates the sympathetic nervous system [19]. In this regard, Elenkov et al. and Straub et al. have proposed that stimulation of sympathetic nerve terminals evokes an inflammatory response

in peripheral organs [40,41]. Catecholamines activate transcription factors, such as nuclear factor-kappa B (NF-κB), in macrophages thereby promoting IL-1β, TNF-α and IL-8 production, which in turn might result in an acute phase response in the liver possibly via α-adrenergic activation [40,42,43]. Therefore, the systemic endothelial activation and inflammation caused by ventilator-induced alveolar stretch may be explained by activation of sympathetic nerve terminals in organs distal to the lung. If so, blockade of adrenergic receptor function will give further insight into the mechanism of distal organ inflammation. Future studies should also aim to develop intervention strategies that prevent simultaneous endothelial activation and inflammation in the lung and distal organs during mechanical ventilation. Such intervention strategies may not only improve the efficacy of mechanical ventilation but could also contribute to prevent MOF.

CONCLUSIONS

We have shown that alveolar stretch imposed by 4 hours of mechanical ventilation did not only provoke *de novo* synthesis of adhesion molecules and recruitment of granulocytes in the lung but also in organs distal to the lung such as liver and kidney, although not the brain. Our results demonstrate that ventilator-induced endothelial activation and inflammation in both the lung and distal organs may be crucial factors in the pathogenesis of MOF.

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CHAPTER

3

Liposome-encapsulated dexamethasone attenuates ventilator-induced lung inflammation: increased efficacy of Fcγ-receptor targeting

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Submitted

ABSTRACT

Background: Systemic glucocorticoid therapy may effectively attenuate lung inflammation but also induce severe side effects. Delivery of glucocorticoids by liposomes could therefore be beneficial. We investigated if liposome-encapsulated dexamethasone inhibits ventilator-induced lung inflammation. Furthermore, we evaluated whether targeting of cellular Fcγ-receptors (FcγRs), by conjugating IgG to liposomes, may improve the efficacy of dexamethasone-liposomes in attenuating granulocyte infiltration, one of the hallmarks of lung inflammation.

Methods: Mice were anesthetized, tracheotomized and mechanically ventilated for 5 hours with either "low" tidal volumes ~7.5 ml/kg (LV_T) or "high" tidal volumes ~15 ml/kg (HV_T). At initiation of ventilation, we intravenously administered either dexamethasone encapsulated in liposomes (Dex-liposomes), dexamethasone encapsulated in IgG-modified liposomes (IgG-Dex-liposomes) or free dexamethasone. Non-ventilated mice served as controls.

Results: Dex-liposomes attenuated granulocyte infiltration and interleukin (IL)-6 mRNA expression after LV_T-ventilation, but not after HV_T-ventilation. Dex-liposomes also down-regulated mRNA expression of IL-1β and keratinocyte-derived chemokine (KC), but not of monocyte chemotatic protein (MCP)-1 in lungs of LV_T and HV_T-ventilated mice. Importantly, IgG-Dex-liposomes inhibited granulocyte influx caused by either LV_T or HV_T-ventilation. IgG-Dex-liposomes diminished IL-1β and KC mRNA expression in both ventilation groups, and IL-6 and MCP-1 mRNA expression in the LV_T-ventilated group. Free dexamethasone prevented granulocyte influx and inflammatory mediator expression induced by LV_T or HV_T-ventilation.

Conclusions: FcγR-targeted IgG-Dex-liposomes are pharmacologically more effective than Dex-liposomes particularly in inhibiting pulmonary granulocyte infiltration. Especially IgG-Dex-liposomes inhibited most parameters of ventilator-induced lung inflammation as effectively as free dexamethasone, with the advantage that liposome-encapsulated dexamethasone will be released locally in the lung thereby preventing systemic side effects.

INTRODUCTION

Mechanical ventilation has the potential to induce or worsen lung injury, a phenomenon referred to as ventilator-induced lung injury (VILI) [1,2]. VILI is characterized by enhanced inflammation, vascular leakage and impaired gas exchange [3]. It has been hypothesized that a ventilator-induced increase in granulocyte infiltration and inflammatory mediator expression in the lung is crucial in the development of pulmonary injury [4-6]. One of the most potent drugs to treat lung inflammation are glucocorticoids, which exert their effects by binding to intracellular glucocorticoid receptors (GRs) [7,8]. After binding, the GR complex migrates from the cytosol to the nucleus where it regulates a host of gene activity, including inhibition of nuclear factor-kappa B (NF-κB) and activator protein (AP)-1 driven expression of inflammatory genes [9].

Previous research in experimental models of VILI showed that synthetic glucocorticoids may attenuate ventilator-induced lung inflammation [10,11]. However, systemic administration of glucocorticoids is associated with severe side effects like increased blood glucose levels [12,13]. Local delivery of glucocorticoids by liposomal formulations could therefore be of therapeutic importance. In this respect, we previously demonstrated that delivery of liposome-encapsulated dexamethasone (Dex-liposomes) inhibits pro-inflammatory gene expression in a murine model of glomerulonephritis without affecting blood glucose levels [14]. Liposomes are valuable drug delivery systems for treatment of VILI as they can act as a depot from which the encapsulated drug will be slowly released to enable prolonged drug exposure at low concentrations [15]. Furthermore, liposomes extravasate into tissues that experience increased capillary permeability [15] which facilitates delivery at sites of inflammation or mechanical stretch.

The present study was designed to examine whether Dex-liposomes are capable of downregulating ventilator-induced lung inflammation. Moreover, we hypothesized that conjugation of IgG to the Dex-liposomes would promote binding of the IgG Fc-fragment on Dex-liposomes to Fcγ-receptors (FcγRs) on macrophages and granulocytes, thereby improving the efficacy of Dex-liposomes to inhibit granulocyte infiltration, one of the hallmarks of lung inflammation. We investigated the effects of dexamethasone encapsulated in liposomes (Dex-liposomes), dexamethasone encapsulated in IgG-modified liposomes (IgG-Dex-liposomes) and free dexamethasone in an established murine model of VILI [16].

MATERIALS AND METHODS

Animals

The animal use and care committees of the University Medical Center Utrecht and Academic Medical Center Amsterdam approved all experiments. Adult male C57Bl6 mice (n=126; Charles River, Maastricht, the Netherlands), weighing 20 to 24 grams, were randomly assigned to different experimental groups.

Study design

Healthy mice (n=108) were exposed to mechanical ventilation as described previously [16]. In short, 6 mice were simultaneously ventilated for 5 hours in a pressure-controlled mode, at a fractional inspired oxygen concentration (FiO_2) of 0.5, inspiration-to-expiration ratio of 1:1 and positive end-expiratory pressure (PEEP) of 2 cmH_2O. Mechanical ventilation was initiated with either an inspiratory pressure of 10 cmH_2O (resulting in "low" tidal volume (V_T) ~7.5 ml/kg; LV_T) or 18 cmH_2O (resulting in "high" V_T ~15 ml/kg; HV_T). Respiratory rate was set at 100 and 50 breaths/min, respectively. Body temperature was kept constant between 36.5 and 37.5°C. Non-ventilated mice (n=18) served as controls (non-ventilated controls, NVC).

Synthesis of liposome-encapsulated dexamethasone

The glucocorticoid dexamethasone was encapsulated in liposomes (Dex-liposomes) or IgG-modified liposomes (IgG-Dex-liposomes) as described previously [14]. Lipids from stock solutions of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, cholesterol, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000] and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000]-maleimide in chloroform/methanol (9:1), were mixed in a molar ratio of 55:40:4:1, dried under reduced nitrogen pressure, dissolved in cyclohexane and lyophilized. The lipids were then hydrated in 10 mM HEPES and 135 mM NaCl, pH 6.7, or, when appropriate, in an aqueous solution of 75 to 100 mg/ml dexamethasone disodium phosphate. The liposomes formed were sized by repeated extrusion (13 times) through polycarbonate filters (Costar; Corning Life Sciences, Acton, MA), pore size 50 nm, using a high-pressure extruder (Lipex, Vancouver, BC, Canada). The rat IgG (Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) was thiolated by *N*-succinimidyl-*S*-acetylthioacetate and coupled to a maleimide group at the distal end of the polyethylene glycol chain. The liposomes were characterized by determining protein content, using mouse IgG as a standard [17] and phospholipid phosphorus content [18]. Total liposomal lipid concentrations were adjusted for the amount of cholesterol present in liposome preparations. The amount of coupled rat IgG was 34.2 µg per µmol of lipid. Particle size was

analyzed by dynamic light scattering using a Nicomp model 370 submicron particle analyzer (Santa Barbara, CA) in the volume weighing model. The diameter of Dex-liposomes was 88.6 nm and that of IgG-Dex-liposomes 103.0 nm. The content of encapsulated dexamethasone disodium-phosphate (Bufa, Hilversum, the Netherlands) was determined after Bligh and Dyer extraction in the resulting methanol/H_2O phase by high-performance liquid chromatography [19]. The amount of encapsulated dexamethasone phosphate was 28.0 µg per µmol of lipid (Dex-liposomes) or 34.8 µg per µmol of lipid (IgG-Dex-liposomes).

Dexamethasone treatment

At initiation of ventilation, we intravenously administered either Dex-liposomes, IgG-Dex-liposomes (0.4 µmol of lipid per animal; 11.2 and 13.9 µg dexamethasone respectively) or free dexamethasone (20 µg per animal; Bufa). Control LV_T and HV_T-ventilated mice received the same volume of sterile saline (vehicle) intravenously.

Hemodynamics and blood gas analysis

After 0, 2.5 and 5 hours, systolic blood pressure and heart rate were non-invasively monitored using a murine tail-cuff system (ADInstruments, Spenbach, Germany). After 5 hours, arterial blood was taken from the carotid artery for blood gas analysis (Rapidlab 865; Bayer, Mijdrecht, the Netherlands).

Histopathology

The left lung was filled with Tissue-Tek® (Sakura Finetek, Zoeterwoude, the Netherlands), snap frozen and cut to 5 µm cryosections using a cryostat. Longitudinal sections were stained with hematoxylin-eosin (H&E; Klinipath, Duiven, the Netherlands).

Tissue homogenate preparation

Lung tissue was pulverized using a liquid nitrogen-cooled mortar and pestle, and divided in several fractions allowing us to perform multiple analyses (as described below).

Myeloperoxidase (MPO) assay

MPO activity was determined as described previously [20]. In short, pulmonary tissue was homogenized in 50 mM HEPES buffer (pH 8.0), centrifuged and pellets were homogenized again in water and 0.5% cetyltrimethylammonium chloride (CTAC; Merck, Darmstadt, Germany). After centrifugation, supernatants were diluted in 10 mM citrate buffer (pH

5.0) and 0.22% CTAC. Substrate solution containing 3 mM 3',5,5'-tetramethylbenzidine dihydrochloride (TMB; Sigma-Aldrich, Steinheim, Germany), 120 μM resorcinol (Merck) and 2.2 mM hydrogen peroxide (H_2O_2) in distilled water was added. Reaction mixtures were incubated for 20 minutes at room temperature and stopped by addition of 4M sulfuric acid (H_2SO_4) followed by determination of optical density at 450 nm. MPO activity of a known amount of MPO units (Sigma-Aldrich) was used as reference. MPO activity was corrected for total protein (BCA protein assay; Pierce Biotechnology, Rockford, IL) using BSA as standard.

Real-time RT-PCR analysis

Total RNA was isolated with TRIzol® reagent (Invitrogen, Paisley, UK). cDNA was synthesized with SuperScript Reverse Transcriptase (Invitrogen). PCR reaction was performed with iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) using primers for interleukin (IL)-1β, IL-6, keratinocyte-derived chemokine (KC) and monocyte chemotatic protein (MCP)-1. PCR product size was verified on gel to confirm appropriate amplification. Data were normalized for expression of internal controls, i.e. the average value of β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPdH). Primers: IL-6, FW ACCgCTATgAAgTTCCTCTC, RV CTCTgTgAAgTCTCCTCTCC; MCP-1 FW ggTCCCTgTCATgCTTCTg, RV CATCTTgCTggTgAATgAgTAg; for other sequences see [20].

Statistical analysis

Data are expressed as mean ± SEM. Blood gas variables (LV_T versus HV_T) were analyzed by independent T-test. All other parameters were analyzed by one-way ANOVA with least significant difference (LSD) post-hoc test. P-values less than 0.05 were considered statistically significant.

RESULTS

Stability of the murine model of VILI

All animals survived the ventilation procedures and were sacrificed after 5 hours of mechanical ventilation. Systolic blood pressures and heart rates were stable throughout the experiment, with no differences between the experimental groups over time (table 1).

Arterial oxygen tension (PaO_2) was reduced in HV_T-ventilated mice in comparison with LV_T-ventilated mice (table 2). In both ventilation groups, carbon dioxide tension ($PaCO_2$), pH and base excess (BE) remained within the physiological range.

Table 1. Hemodynamic characteristics over 5 hours of mechanical ventilation

	LV_T	HV_T
$HR_{t=0\ hr}$	358 ± 10	376 ± 18
$HR_{t=2,5\ hr}$	385 ± 12	338 ± 16
$HR_{t=5\ hr}$	408 ± 12	350 ± 8
$BP_{t=0\ hr}$	103 ± 5	99 ± 7
$BP_{t=2,5\ hr}$	73 ± 5	72 ± 3
$BP_{t=5\ hr}$	75 ± 5	73 ± 4

Data are expressed as mean ± SEM (LV_T n=10, HV_T n=10). LV_T, HV_T = mechanically ventilated with low or high tidal volumes; HR= heart rate in beats/minute, BP= systolic blood pressure in mmHg.

Tabel 2. Arterial blood gas analysis after 5 hours of mechanical ventilation

	LV_T	HV_T
PaO_2	225.3 ± 13.6	175.8 ± 11.0**
$PaCO_2$	31.0 ± 2.1	35.8 ± 2.2
pH	7.52 ± 0,03	7.50 ± 0,02
BE	2.33 ± 0.75	3.43 ± 0.62

Data are expressed as mean ± SEM (LV_T n=18, HV_T n=20). ** $p<0.01$ versus LV_T. LV_T, HV_T = mechanically ventilated with low or high tidal volumes; PaO_2 = partial pressure of arterial oxygen in mmHg; $PaCO_2$ = partial pressure of arterial carbon dioxide in mmHg; BE = base excess in mmol/l.

Effect of Dex-liposomes and IgG-Dex-liposomes on pulmonary architecture

Lung sections were stained for H&E to analyze histopathological changes after mechanical ventilation (figure 1). We observed that both LV_T and HV_T-ventilation (vehicle-treatment) induced damage to pulmonary architecture compared to NVC, i.e. thickening of the alveolar wall and infiltration of granulocytes. Administration of Dex-liposomes, IgG-Dex-liposomes or free dexamethasone at the initiation of ventilation preserved pulmonary architecture during 5 hours of mechanical ventilation.

Effect of Dex-liposomes and IgG-Dex-liposomes on ventilator-induced granulocyte infiltration

To assess ventilator-induced lung inflammation, we determined influx of granulocytes into pulmonary tissue. Granulocyte infiltration was quantified by measuring MPO activity in total lung homogenates. MPO activity was significantly increased after LV_T and HV_T-ventilation in comparison with NVC (figure 2), which correlated with the margination of granulocytes to the blood vessel wall observed in lung sections of LV_T and HV_T-ventilated mice (figure 1).

Next, we investigated if administration of Dex-liposomes at the initiation of ventilation was capable of inhibiting lung inflammation induced by 5 hours of LV_T or HV_T-ventilation.

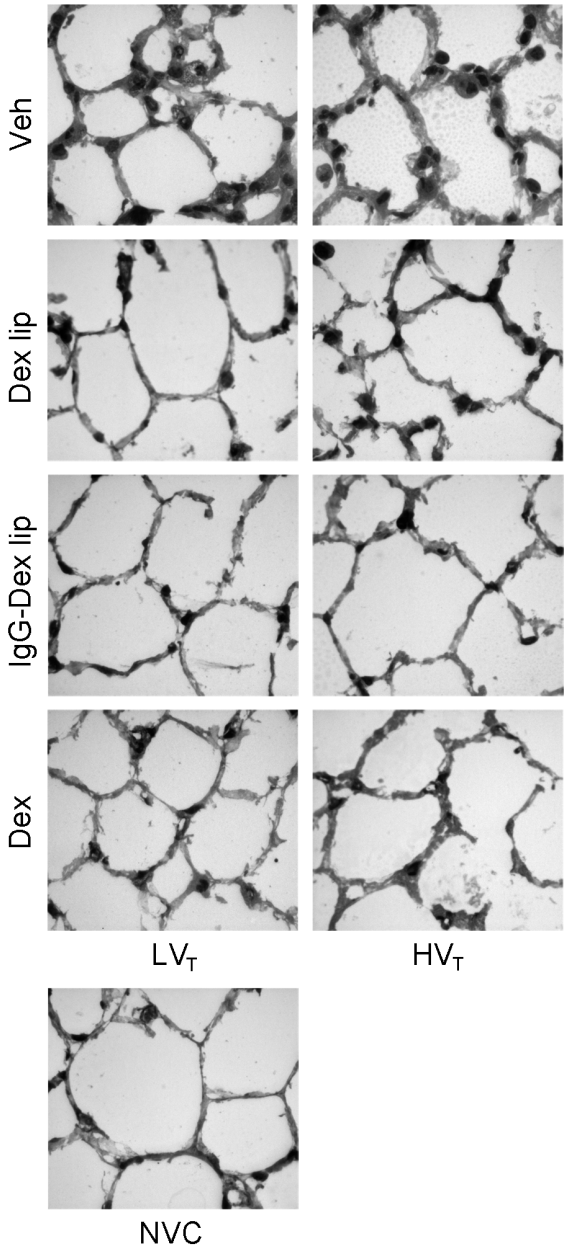

Figure 1: Effect of Dex-liposomes, IgG-Dex-liposomes and free dexamethasone on changes in lung histology induced by mechanical ventilation. (See color figure on pg. 196.)
Lung sections were stained with hematoxylin-eosin (H&E) to analyze lung architecture and presence of granulocytes in pulmonary tissue. Magnification ×500.
NVC = non-ventilated controls; LV_T, HV_T = mechanically ventilated with low or high tidal volumes; Veh, Dex lip, IgG-Dex lip, Dex = intravenously treated with either vehicle (sterile saline), liposomes containing dexamethasone, IgG-modified liposomes containing dexamethasone, or free dexamethasone.

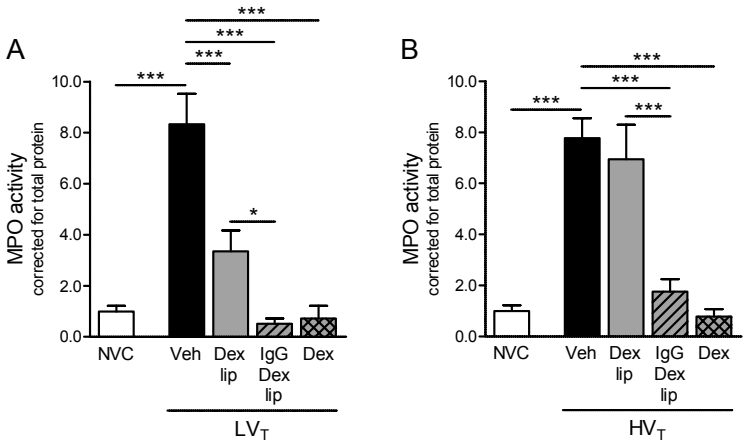

Figure 2: Effect of Dex-liposomes, IgG-Dex-liposomes and free dexamethasone on granulocyte influx induced by mechanical ventilation.

A-B: In total lung homogenates, myeloperoxidase (MPO) activity was determined as a measure of granulocyte infiltration. Levels were normalized for total protein levels. Data are expressed as mean ± SEM, and depicted relative to NVC. A/B: NVC n=18/18, Veh n=20/14, Dex lip n=15/14, IgG-Dex lip n=10/9, Dex n=7/7. * $p<0.05$, *** $p<0.001$.

NVC = non-ventilated controls; LV_T, HV_T = mechanically ventilated with low or high tidal volumes; Veh, Dex lip, IgG-Dex lip, Dex = intravenously treated with either vehicle (sterile saline), liposomes containing dexamethasone, IgG-modified liposomes containing dexamethasone, or free dexamethasone.

Treatment with Dex-liposomes attenuated MPO activity in mice exposed to LV_T-ventilation (figure 2a) although MPO activity was still above baseline level in these mice ($p<0.05$ versus NVC). In mice exposed to HV_T-ventilation, however, no inhibitory effect of Dex-liposomes on MPO activity was observed (figure 2b). In contrast, treatment with IgG-Dex-liposomes effectively inhibited granulocyte infiltration in both LV_T and HV_T-ventilated mice. Furthermore, IgG-Dex-liposomes were as effective as free dexamethasone in downregulating MPO activity in lungs of LV_T and HV_T-ventilated mice. H&E staining of lung sections confirmed the quantitative measures for granulocyte influx after treatment with Dex-liposomes, IgG-Dex-liposomes or free dexamethasone (figure 1).

Effect of Dex-liposomes and IgG-Dex-lipsomes on ventilator-induced cytokine expression

To examine the effect of LV_T and HV_T-ventilation on cytokine expression, we determined *de novo* synthesis of the prototypic pro-inflammatory cytokines IL-1β and IL-6 in total lung homogenates. Compared to NVC, both ventilation strategies induced mRNA expression of IL-1β (figures 3a and b) and IL-6 (figures 3c and d).

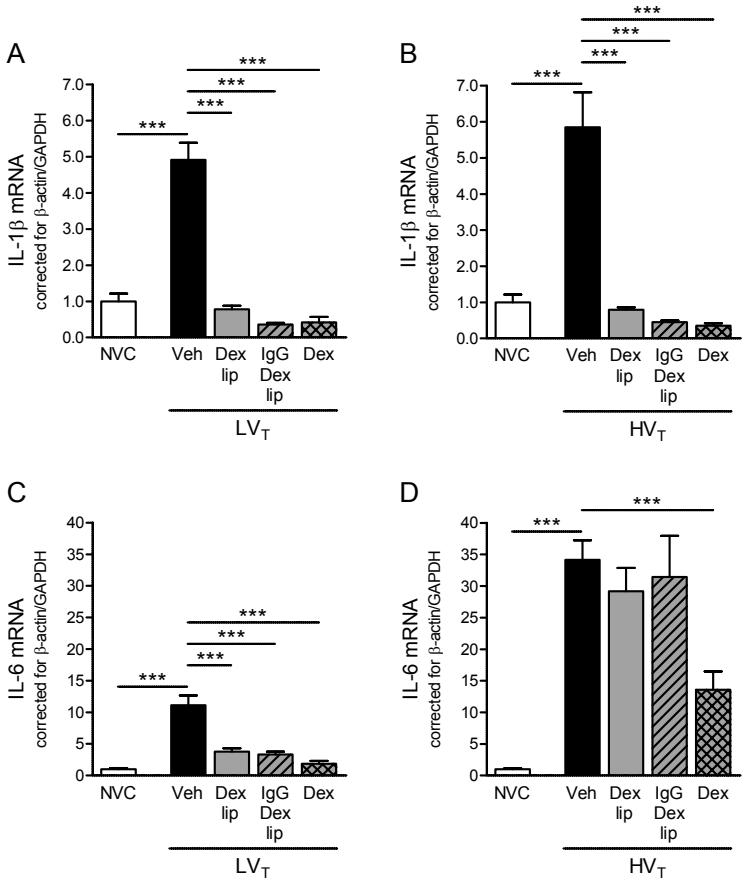

Figure 3: Effect of Dex-liposomes, IgG-Dex-liposomes and free dexamethasone on cytokine mRNA expression induced by mechanical ventilation.

A-B: In total lung homogenates, mRNA expression of interleukin (IL)-1β was determined by real-time RT-PCR. **C-D:** In addition, mRNA expression of IL-6 was determined. Levels were normalized for expression of internal controls, i.e. the average value of β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPdH). Data are expressed as mean ± SEM, and depicted relative to NVC. A/B: NVC n=15/15, Veh n=22/17, Dex lip n=15/12, IgG-Dex lip n=10/8, Dex n=7/7; C/D: NVC n=15/15, Veh n=21/18, Dex lip n=15/13, IgG-Dex lip n=10/10, Dex n=7/7. *** $p<0.001$.

NVC = non-ventilated controls; LV_T, HV_T = mechanically ventilated with low or high tidal volumes; Veh, Dex lip, IgG-Dex lip, Dex = intravenously treated with either vehicle (sterile saline), liposomes containing dexamethasone, IgG-modified liposomes containing dexamethasone, or free dexamethasone.

Dex-liposomes and IgG-Dex-liposomes attenuated IL-1β mRNA expression after LV_T or HV_T-ventilation. IL-6 mRNA levels were downregulated in LV_T-ventilated mice, while both liposomal formulations were fully devoid of IL-6 mRNA inhibitory activity in HV_T-ventilated mice. Administration of free dexamethasone prevented the increase in IL-1β and IL-6 mRNA expression in lungs of LV_T and HV_T-ventilated mice, although IL-6 mRNA expression was still above baseline level in HV_T-ventilated mice ($p<0.05$ versus NVC, figure 3d).

Effect of Dex-liposomes and IgG-Dex-lipsomes on ventilator-induced chemokine expression

To study the effect of LV_T and HV_T-ventilation on *de novo* synthesis of chemokines, we determined KC and MCP-1 mRNA expression in total lung homogenates. We observed that mRNA expression of KC (figures 4a and b) and MCP-1 (figures 4c and d) was increased after LV_T and HV_T ventilation.

Treatment with Dex-liposomes downregulated KC mRNA expression induced by LV_T or HV_T-ventilation. However, Dex-liposomes did not affect MCP-1 mRNA levels in LV_T-ventilated mice while they enhanced MCP-1 mRNA levels in HV_T-ventilated mice (204.5%; $p<0.001$ versus HV_T vehicle, figure 4d). IgG-Dex-liposomes diminished KC mRNA expression after both LV_T and HV_T-ventilation and MCP-1 mRNA expression only after LV_T-ventilation. Administration of free dexamethasone inhibited KC and MCP-1 mRNA levels in lungs of LV_T and HV_T-ventilated mice.

DISCUSSION

The present study was designed to evaluate whether liposome-encapsulated dexamethasone attenuated lung inflammation induced by mechanical ventilation. Our major finding is that FcγR-targeted IgG-Dex-liposomes are pharmacologically more effective than Dex-liposomes particularly in preventing influx of granulocytes, a major hallmark of VILI. We demonstrated that treatment with IgG-Dex-liposomes successfully protected against granulocyte influx induced by LV_T or HV_T-ventilation, which was accompanied by inhibition of IL-1β and KC mRNA expression in both ventilation groups, and IL-6 and MCP-1 mRNA expression in the LV_T-ventilated group. Moreover, IgG-Dex-liposomes inhibited most parameters of ventilator-induced lung inflammation as effectively as free dexamethasone. In contrast, Dex-liposomes only attenuated IL-1β and KC mRNA during both ventilation strategies and IL-6 mRNA and granulocyte influx during LV_T-ventilation. All administration forms of dexamethasone preserved lung architecture during mechanical ventilation.

Both LV_T and HV_T-ventilation induced inflammation in pulmonary tissue as measured by increased granulocyte infiltration and cytokine/chemokine mRNA expression. Previous studies described that the combined effects of high PaO_2 levels and mechanical ventilation worsen VILI [21,22]. Since we used moderate hyperoxia (FiO_2 of 0.5) in our experimental model of VILI, it cannot be excluded that the higher oxygen levels may aggravate the stretch-induced inflammatory response in the lung. However, we demonstrated before that hyperoxia (FiO_2 of 1.0) as such did not lead to pulmonary inflammation [20]. Therefore, it is tempting to speculate that the moderate hyperoxia in our present study will not be the primary cause of lung inflammation during mechanical ventilation.

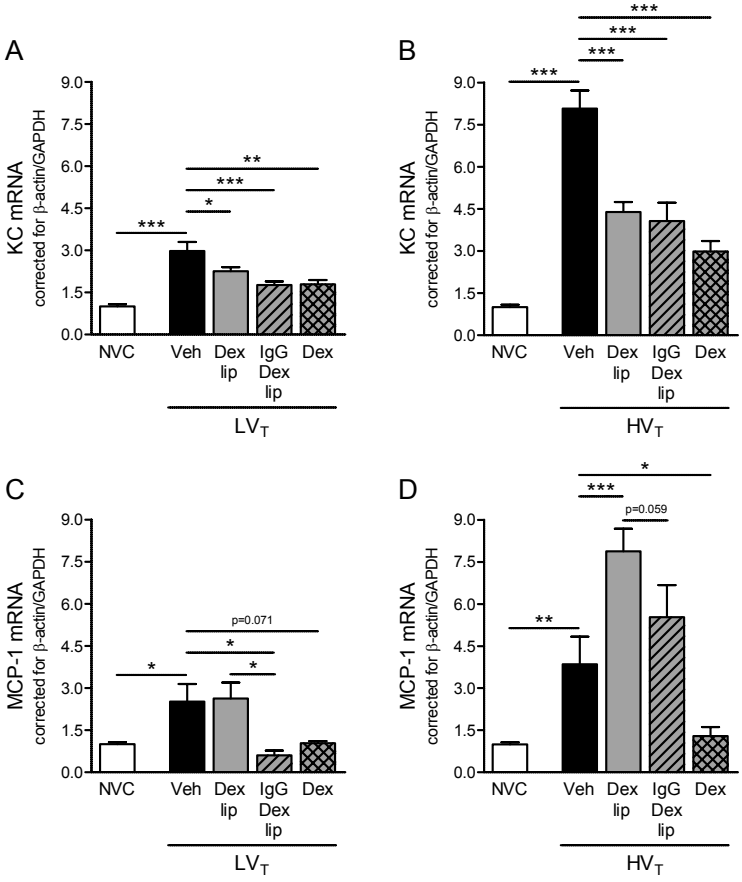

Figure 4: Effect of Dex-liposomes, IgG-Dex-liposomes and free dexamethasone on chemokine mRNA expression induced by mechanical ventilation.

A-B: In total lung homogenates, mRNA expression of keratinocyte-derived chemokine (KC) was determined by real-time RT-PCR. **C-D:** In addition, mRNA expression of monocyte chemotactic protein (MCP)-1 was determined. Levels were normalized for expression of internal controls, i.e. the average value of β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPdH). Data are expressed as mean ± SEM, and depicted relative to NVC. A/B: NVC n=15/15, Veh n=20/17, Dex lip n=15/13, IgG-Dex lip n=10/9, Dex n=8/7; C/D: NVC n=15/15, Veh n=21/16, Dex lip n=15/13, IgG-Dex lip n=10/9, Dex n=8/7. * p<0.05, ** p<0.01, *** p<0.001.

NVC = non-ventilated controls; LV_T, HV_T = mechanically ventilated with low or high tidal volumes; Veh, Dex lip, IgG-Dex lip, Dex = intravenously treated with either vehicle (sterile saline), liposomes containing dexamethasone, IgG-modified liposomes containing dexamethasone, or free dexamethasone.

In agreement with prior reports [10,11], we showed that free dexamethasone successfully protects against lung inflammation induced by 5 hours of mechanical ventilation. Previously, we described in a murine model of glomerulonephritis that liposome-encapsulated dexamethasone prevented the occurrence of hyperglycemia [14], one of the first clinically

relevant side effects of free dexamethasone treatment [12,13]. The main reason for using drug delivery systems in treatment of lung inflammation is thus that liposome-encapsulated glucocorticoids may significantly inhibit inflammatory responses without inducing severe systemic side effects of the free drug.

An important feature of liposomes is their preferential extravasation at sites with increased capillary permeability [15]. As ventilator-induced stretch of lung tissue enhances vascular permeability [23-25], liposomes may accumulate in cells of the lung and slowly release their encapsulated dexamethasone locally. Indeed, we observed that administration of Dex-liposomes significantly inhibited IL-1β and KC mRNA expression in lungs of LV_T and HV_T-ventilated mice, and IL-6 mRNA expression in lungs of LV_T-ventilated mice. More importantly, Dex-liposomes diminished granulocyte infiltration induced by LV_T-ventilation.

Even though Dex-liposomes were capable of attenuating important parameters of VILI, especially in LV_T-ventilated mice, they were not as effective as free dexamethasone in preventing granulocyte infiltration. Since granulocytes are known to be important in the pathogenesis of VILI [26], active delivery of the drug into these circulating granulocytes might be advantageous. Therefore, we hypothesized that IgG-Dex-liposomes may be more efficient in inhibiting ventilator-induced lung inflammation than Dex-liposomes due to interaction with FcγRs on activated granulocytes and macrophages [27]. We found that IgG-Dex-liposomes significantly inhibited granulocyte influx in lungs of both LV_T and HV_T-ventilated mice whereas Dex-liposomes only attenuated granulocyte infiltration in lungs of LV_T-ventilated mice. Based on this notion, we propose that IgG-Dex-liposomes interact more efficiently with granulocytes in the systemic circulation or in the marginal zone of vessels thereby preventing granulocyte activation and infiltration into lung tissue. The observation that glucocorticoids suppress granulocyte activation and recruitment into the alveolar space [11] supports our current findings. Moreover, (IgG)-Dex-liposomes may also be internalized more efficiently by macrophages in the lung, where they primarily prevent *de novo* synthesis of IL-1β and KC.

Previously, Suntres and Shek described that liposome-encapsulated dexamethasone diminished neutrophil activation and infiltration in a rat model of lipopolysaccharide (LPS)-induced acute lung injury (ALI) [28]. Moreover, these authors demonstrated that liposome-encapsulated dexamethasone was even more effective in downregulating LPS-induced lung inflammation than free dexamethasone. Here we show, however, that IgG-Dex-liposomes inhibited granulocyte influx in lungs of LV_T and HV_T-ventilated mice as efficiently as free dexamethasone. An explanation for this discrepancy in efficacy might be that Suntres and Shek investigated the effects of liposome-encapsulated and free dexamethasone at 24 hours after LPS-challenge [28]. Because liposomes are considered as slow-release systems [29], it may well be that liposomes become more effective than free dexamethasone after longer periods of time. Furthermore, it is important to note that the underlying mechanisms of LPS- and ventilator-induced lung inflammation are likely to be different as well, especially with respect to the consequences of mechanical stretch on lung tissue. Taken together, our

present data show that liposome-encapsulated dexamethasone may effectively prevent infiltration of granulocytes and *de novo* synthesis of pro-inflammatory cytokines/chemokines, in particular during LV_T-ventilation.

Interestingly, Dex-liposomes and IgG-Dex-liposomes did not influence IL-6 and MCP-1 mRNA expression induced by HV_T-ventilation. These results may indicate that the target cells of our liposomal formulations may not be the only source of IL-6 and MCP-1 production. *In vitro* studies have demonstrated that alveolar epithelial and capillary endothelial cells are also activated upon mechanical stretch, in a stretch-amplitude dependent matter, thereby releasing inflammatory mediators into the surrounding pulmonary tissue [30,31]. Iwaki et al. demonstrated that high cyclic stretch of microvascular endothelial cells led to increased production of IL-6 and MCP-1 [30]. In agreement with these previous findings, our study shows that IL-6 and MCP-1 mRNA levels were elevated in HV_T-ventilated mice which were exposed to high levels of mechanical stretch. It is therefore tempting to speculate that alveolar epithelial and capillary endothelial cells will not internalize the liposomal formulations and therefore be responsible for the lack of IL-6 and MCP-1 downregulation after liposome treatment. The fact that liposome-encapsulated dexamethasone was effective in inhibiting IL-1β and KC, which are primarily produced by alveolar macrophages [32], supports this explanation. Future experiments are needed to confirm these speculations and to obtain further insights into the working mechanism of Dex-liposomes and IgG-Dex-liposomes.

In conclusion, our study shows for the first time that liposomal formulation of the glucocorticoid dexamethasone prevents important parameters of ventilator-induced lung inflammation. Importantly, we observed that the selective targeting of Dex-liposomes to cells expressing FcγRs, by conjugating IgG to the liposomes, markedly improved their efficacy. In this respect, especially IgG-Dex-liposomes inhibited most parameters of ventilator-induced lung inflammation as effective as free dexamethasone with the advantage that liposome-encapsulated dexamethasone will be released locally in the lung thereby preventing systemic side effects. In view of our data, we suggest that IgG-Dex-liposomes may be an attractive therapeutic strategy to treat critically ill patients diagnosed with serious inflammatory lung diseases like VILI.

CONCLUSIONS

FcγR targeted IgG-Dex-liposomes are pharmacologically more effective than Dex-liposomes in particular with respect to inhibition of granulocyte infiltration induced by either LV_T or HV_T-ventilation. Especially IgG-Dex-liposomes downregulate most parameters of ventilator-induced lung inflammation as efficiently as free dexamethasone.

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CHAPTER

4

Downregulation of lung inflammation by dexamethasone does not prevent ventilator-induced lung injury

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Submitted

ABSTRACT

Background: The appreciation that granulocytes and inflammatory mediators are important in the development of ventilator-induced lung injury (VILI) has led to the suggestion that anti-inflammatory agents may prevent impairment of lung function. The present study was designed to investigate whether dexamethasone, a widely used anti-inflammatory synthetic glucocorticoid, protects against vascular leakage and impaired gas exchange induced by mechanical ventilation.

Methods: Healthy mice were anesthetized, tracheotomized and mechanically ventilated for 5 hours with either an inspiratory pressure of 10 cmH_2O (resulting in "low" tidal volumes (V_T) ~7.5 ml/kg; LV_T) or 18 cmH_2O (resulting in "high" V_T ~15 ml/kg; HV_T). Dexamethasone was intravenously administered at initiation of ventilation. Non-ventilated mice served as controls.

Results: Both LV_T and HV_T-ventilation increased expression of inflammatory mediators (keratinocyte-derived chemokine (KC), macrophage inflammatory protein (MIP)-2, monocyte chemotactic protein (MCP)-1, interleukin (IL)-1β, IL-6, E-selectin) which was accompanied by marked granulocyte infiltration into pulmonary tissue. Vascular endothelial growth factor (VEGF) expression was only enhanced in HV_T-ventilated mice. Furthermore, mechanical ventilation increased alveolar protein levels and pulmonary wet-to-dry ratios in both ventilation groups and reduced PaO_2/FiO_2 ratios in the HV_T-ventilation group. Importantly, treatment with dexamethasone inhibited the inflammatory response and VEGF expression in lung tissue but did not prevent vascular leakage and impaired gas exchange induced by mechanical ventilation. Dexamethasone even aggravated ventilator-induced effects on vascular leakage in HV_T-ventilated mice.

Conclusions: Although treatment with dexamethasone inhibits pulmonary inflammation and VEGF expression, it does not protect against vascular leakage and impaired gas exchange induced by mechanical ventilation.

INTRODUCTION

It has been appreciated that mechanical ventilation as such may provoke progressive damage to pulmonary tissue, known as ventilator-induced lung injury (VILI) [1,2]. The main characteristics of VILI are the release of inflammatory mediators (i.e. cytokines, chemokines) accompanied by granulocyte infiltration and the disruption of alveolar-capillary barriers, eventually resulting in pulmonary dysfunction [3]. At present, it is thought that a ventilator-induced inflammatory response in the lung may precede pulmonary injury [4-6]. Activated granulocytes cause oxidative stress and protease activity in the alveoli, subsequently inducing severe disruption of pulmonary epithelial-endothelial barriers and leading to impaired gas exchange [7-9]. The awareness that granulocytes and inflammatory mediators are involved in the pathogenesis of severe inflammatory disorders like acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) has led to the application of anti-inflammatory agents like glucocorticoids [10,11].

Glucocorticoids are a class of steroid hormones that exert their anti-inflammatory and immunosuppressive effects by binding to intracellular glucocorticoid receptors (GRs). After binding, the GR complex migrates from the cytosol to the nucleus where it regulates a host of gene activity, including inhibition of nuclear factor-kappa B (NF-κB) and activator protein (AP)-1 driven expression of inflammatory genes [12]. In addition, it has been described that glucocorticoids suppress granulocyte recruitment and activation, preserve endothelial cell integrity and control vascular permeability [13,14]. The efficacy of using synthetic glucocorticoids to treat ALI/ARDS in critically ill patients is still under debate [15-17]. In experimental models of VILI, however, glucocorticoid pre-treatment has shown promising results on reducing lung injury [13,18]. It should be noted, though, that duration of mechanical ventilation was rather short in these studies.

The present study was designed to investigate the anti-inflammatory action of dexamethasone in mice mechanically ventilated for 5 hours with low or high tidal volumes. We applied a mild model of VILI using ventilation strategies with clinically relevant ventilator settings thereby preventing shock, metabolic acidosis and substantial damage to pulmonary architecture [19]. We hypothesized that downregulation of lung inflammation by dexamethasone would protect ventilated mice against important hallmarks of VILI, such as alveolar-capillary permeability, pulmonary edema (i.e. vascular leakage) and impaired gas exchange.

MATERIALS AND METHODS

Animals

Experiments were approved by the animal use and care committees of the University Medical Center Utrecht and Academic Medical Center Amsterdam. Adult male C57Bl6 mice (n=140; Charles River, Maastricht, the Netherlands), weighing 20 to 24 grams, were randomly assigned to different experimental groups.

Study design

Mice (n=108) were exposed to mechanical ventilation as described previously [19]. In short, 6 mice were simultaneously ventilated for 5 hours in a pressure-controlled mode, at a fractional inspired oxygen concentration (FiO_2) of 0.5, inspiration-to-expiration ratio of 1:1 and positive end-expiratory pressure of 2 cmH_2O. Mechanical ventilation was initiated with either an inspiratory pressure of 10 cmH_2O (resulting in "low" tidal volume (V_T) ~7.5 ml/kg; LV_T) or 18 cmH_2O (resulting in "high" V_T ~15 ml/kg; HV_T). Respiratory rate was set at 100 and 50 breaths/min, respectively. Body temperature was kept constant between 36.5 and 37.5°C. Non-ventilated mice (n=32) served as controls (non-ventilated controls, NVC).

Dexamethasone treatment

Dexamethasone (0.8 mg/kg; Bufa, Hilversum, the Netherlands) was intravenously administered at initiation of ventilation. Control LV_T or HV_T-ventilated mice received the same volume of sterile saline (vehicle) intravenously.

Hemodynamics and blood gas analysis

After 0, 2.5 and 5 hours, systolic blood pressure and heart rate were non-invasively monitored using a murine tail-cuff system (ADInstruments, Spenbach, Germany). After 5 hours, arterial blood was taken from the carotid artery for blood gas analysis (Rapidlab 865; Bayer, Mijdrecht, the Netherlands).

Bronchoalveolar lavage

The right lung was lavaged by instilling 3x 0.5 ml sterile saline into the trachea. Differential counts were done on bronchoalveolar lavage fluid (BALf) cytospin preparations stained with Giemsa (Diff-Quick; Dade Behring AG, Düdingen, Switzerland). Cell-free supernatant was used to measure total protein (BCA protein-assay; Pierce Biotechnology, Rockford, IL).

Wet-to-dry ratio

The left lung was weighed, dried for 3 days in a 65°C stove and weighed again.

Histopathology

The left lung was filled with Tissue-Tek® (Sakura Finetek, Zoeterwoude, the Netherlands), snap frozen and cut to 5 µm cryosections using a cryostat. Longitudinal sections were stained with hematoxylin-eosin (H&E; Klinipath, Duiven, the Netherlands).

Tissue homogenate preparation

Lung tissue was pulverized using a liquid nitrogen-cooled mortar and pestle, and divided in several fractions allowing us to perform multiple analyses (as described below).

Real-time RT-PCR analysis

Total RNA was isolated with TRIzol® reagent (Invitrogen, Paisley, UK). cDNA was synthesized with SuperScript Reverse Transcriptase (Invitrogen). PCR reaction was performed with iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) using primers for keratinocyte-derived chemokine (KC), interleukin (IL)-1β and E-selectin; for primer sequences see [20]. PCR product size was verified on gel to confirm appropriate amplification. Data were normalized for expression of internal controls, i.e. the average value of β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPdH).

Multiplex cytokine analysis

125 µg protein was analyzed for KC, macrophage inflammatory protein (MIP)-2, monocyte chemotactic protein (MCP)-1, IL-1β, IL-6, IL-10 and vascular endothelial growth factor (VEGF) by multiplex cytokine assay using a Luminex analyzer (Bio-Rad) according to manufacturer's instructions (multiplex mouse cytokine, R&D systems, Minneapolis, MN).

Myeloperoxidase (MPO) assay

MPO activity was determined as described previously [20]. In short, pulmonary tissue was homogenized in 50 mM HEPES buffer (pH 8.0), centrifuged and pellets were homogenized again in water and 0.5% cetyltrimethylammonium chloride (CTAC; Merck, Darmstadt, Germany). After centrifugation, supernatants were diluted in 10 mM citrate buffer (pH 5.0) and 0.22% CTAC. Substrate solution containing 3 mM 3',5,5'-tetramethylbenzidine

dihydrochloride (TMB; Sigma-Aldrich, Steinheim, Germany), 120 µM resorcinol (Merck) and 2.2 mM hydrogen peroxide (H_2O_2) in distilled water was added. Reaction mixtures were incubated for 20 minutes at room temperature and stopped by addition of 4M sulfuric acid (H_2SO_4) followed by determination of optical density at 450 nm. MPO activity of a known amount of MPO units (Sigma-Aldrich) was used as reference. MPO activity was corrected for total protein (BCA protein assay; Pierce) using BSA as standard.

Statistical analysis

Data are expressed as mean ± SEM. Blood gas variables (LV_T versus HV_T) were analyzed by independent T-test. All other parameters were analyzed by ANOVA with least significant difference (LSD) post-hoc test. P-values less than 0.05 were considered statistically significant.

RESULTS

Stability of the murine model of VILI

All mice survived the LV_T and HV_T-ventilation procedures and were sacrificed after 5 hours. Systolic blood pressures and heart rates were stable throughout the experiment, with no differences between experimental groups (table 1).

Arterial oxygen tension (PaO_2) was significantly lower in HV_T-ventilated mice as compared to LV_T-ventilated mice (table 2). In both ventilation groups, carbon dioxide tension ($PaCO_2$), pH and base excess (BE) remained within the physiological range.

Table 1. Hemodynamic characteristics over 5 hours of mechanical ventilation

	LV_T	HV_T
$HR_{t=0\ hr}$	365 ± 12	387 ± 13
$HR_{t=2,5\ hr}$	375 ± 10	345 ± 13
$HR_{t=5\ hr}$	402 ± 11	359 ± 13
$BP_{t=0\ hr}$	106 ± 6	104 ± 6
$BP_{t=2,5\ hr}$	73 ± 6	73 ± 4
$BP_{t=5\ hr}$	73 ± 5	76 ± 5

Data are expressed as mean ± SEM (LV_T n=11-16, HV_T n=14-16). LV_T, HV_T = mechanically ventilated with low or high tidal volumes; HR= heart rate in beats/minute; BP= systolic blood pressure in mmHg.

Table 2. Arterial blood gas analysis after 5 hours of mechanical ventilation

	LV_T	HV_T
PaO_2	224.6 ± 18.3	172.0 ± 12.6*
$PaCO_2$	30.4 ± 2.6	33.6 ± 2.0
pH	7.54 ± 0,03	7.52 ± 0,03
BE	3.14 ± 1.10	3.88 ± 1.13

Data are expressed as mean ± SEM (LV_T n=16, HV_T n=15). * $p<0.05$ versus LV_T. LV_T, HV_T = mechanically ventilated with low or high tidal volumes; PaO_2 = partial pressure of arterial oxygen in mmHg; $PaCO_2$ = partial pressure of arterial carbon dioxide in mmHg; BE = base excess in mmol/l.

Effect of dexamethasone treatment on ventilator-induced mRNA expression of chemokines, cytokines and adhesion molecules

To determine whether dexamethasone treatment at initiation of ventilation suppressed lung inflammation induced by 5 hours of LV_T or HV_T-ventilation, we studied *de novo* synthesis of the chemokine KC, pro-inflammatory cytokine IL-1β and adhesion molecule E-selectin. We observed that both ventilation strategies induced KC, IL-1β and E-selectin mRNA expression compared to NVC (figures 1a to c). Moreover, HV_T-ventilation enhanced mRNA expression of these inflammatory mediators in comparison with LV_T-ventilation (KC, E-selectin $p<0.001$; IL-1β $p<0.05$). Treatment with dexamethasone inhibited inflammatory gene expression after 5 hours of LV_T or HV_T-ventilation.

Effect of dexamethasone treatment on ventilator-induced protein expression of chemokines and cytokines

Next, we investigated whether dexamethasone treatment influenced protein expression of inflammatory mediators in lungs of LV_T and HV_T-ventilated mice. Compared to NVC, both LV_T and HV_T-ventilation induced expression of the chemokines KC and MIP-2 protein in pulmonary tissue (figures 2a and b). MCP-1 protein expression tended to increase only in lungs of HV_T-ventilated mice ($p=0.075$, figure 2c). Protein expression of the pro-inflammatory cytokine IL-1β was increased in lungs of both LV_T and HV_T-ventilated mice, whereas IL-6 was only enhanced in lungs of HV_T-ventilated mice (figures 2d and e). In addition, we observed that HV_T-ventilation tended to augment IL-6 protein expression in comparison with LV_T-ventilation ($p=0.062$, figure 2e). Treatment with dexamethasone diminished protein expression of chemokines and cytokines induced by mechanical ventilation (figure 2). In lungs of HV_T-ventilated mice, however, the decrease in MIP-2 protein expression did not reach statistical significance ($p=0.090$, figure 2b). The anti-inflammatory cytokine IL-10 was below detection level in all experimental groups.

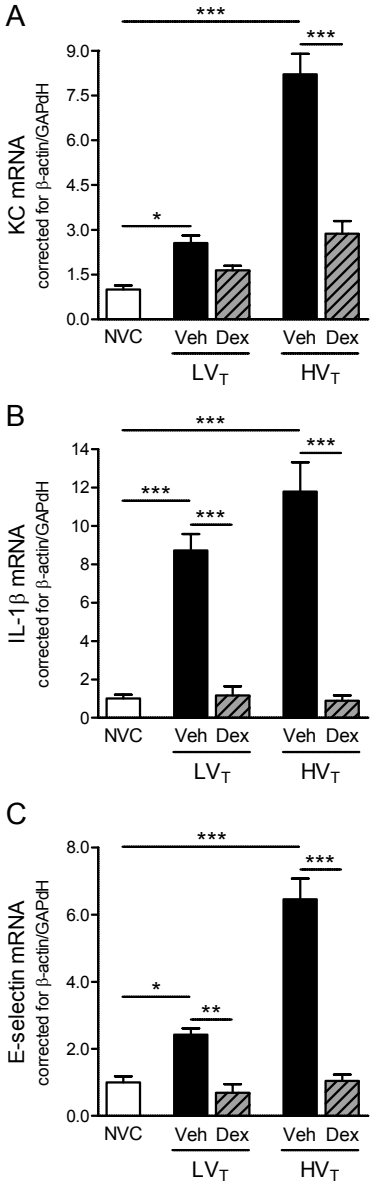

Figure 1: Dexamethasone treatment decreases mRNA expression of inflammatory mediators.
A-C: In total lung homogenates, mRNA expression of the chemoattractant keratinocyte-derived chemokine (KC), pro-inflammatory cytokine interleukin (IL)-1β and adhesion molecule E-selectin was determined by real-time RT-PCR. Levels were normalized for expression of internal controls, i.e. the average value of β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPdH). Data are expressed as mean ± SEM, and depicted relative to NVC. NVC n=7-8, LV_T Veh n=14-15, LV_T Dex n=6, HV_T Veh n=12-13, HV_T Dex n=6. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.
NVC = non-ventilated controls; LV_T, HV_T = mechanically ventilated with low or high tidal volumes; Veh, Dex = intravenously treated with either vehicle (sterile saline) or dexamethasone.

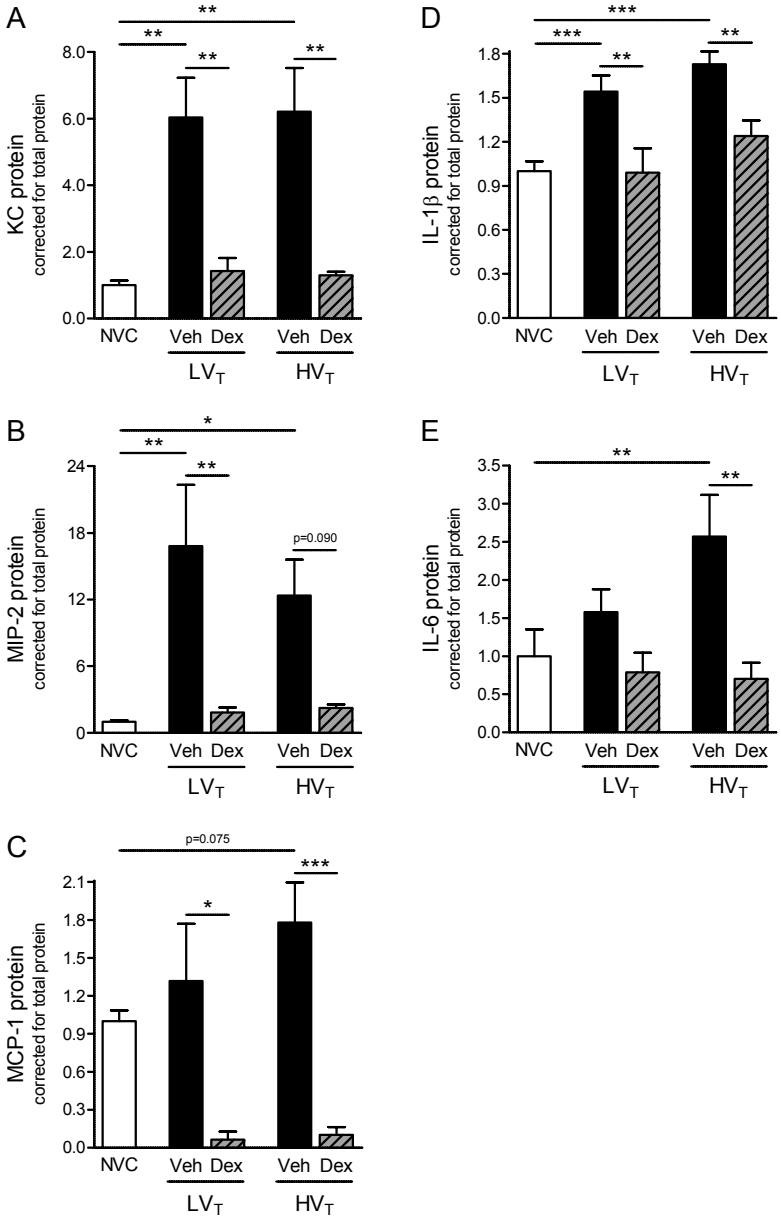

Figure 2: Dexamethasone treatment decreases protein expression of inflammatory mediators.
A-E: In total lung homogenates, protein expression of the chemoattractants keratinocyte-derived chemokine (KC), macrophage inflammatory protein (MIP)-2, monocyte chemotactic protein (MCP)-1 and pro-inflammatory cytokines interleukin (IL)-1β, IL-6 was determined by multiplex cytokine analysis. Data are expressed as mean ± SEM, and depicted relative to NVC. NVC n=7-8, LV_T Veh n=9-10, LV_T Dex n=6, HV_T Veh n=9-10, HV_T Dex n=5-6. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.
NVC = non-ventilated controls; LV_T, HV_T = mechanically ventilated with low of high tidal volumes; Veh, Dex = intravenously treated with either vehicle (sterile saline) or dexamethasone.

Effect of dexamethasone treatment on ventilator-induced infiltration of granulocytes

To assess whether dexamethasone treatment affected granulocyte infiltration induced by mechanical ventilation, we measured MPO activity in total lung homogenates (i.e. granulocyte infiltration into lung tissue) and counted neutrophils on BALf cytospin preparations (i.e. granulocyte exudation into alveolar space). Both LV_T and HV_T-ventilation enhanced MPO activity in comparison with NVC, whereas only HV_T-ventilation increased BALf neutrophil numbers (figures 3a and b). Furthermore, BALf neutrophil numbers were significantly higher when comparing HV_T-ventilated mice with LV_T-ventilated mice ($p<0.05$, figure 3b). Treatment with dexamethasone completely abolished the granulocyte infiltration in pulmonary tissue and granulocyte exudation into the alveolar space induced by LV_T or HV_T-ventilation. In addition, we stained lung sections for H&E to analyze histopathological changes after mechanical ventilation. Figure 3c illustrates that both LV_T and HV_T-ventilation caused margination of granulocytes to the blood vessel wall. Especially HV_T-ventilation induced damage to pulmonary architecture. Dexamethasone administration prevented granulocyte margination in lung tissue of LV_T and HV_T-ventilated mice, confirming the quantitative measures for granulocyte influx. Moreover, we observed that dexamethasone preserved pulmonary architecture during mechanical ventilation.

Effect of dexamethasone treatment on ventilator-induced protein expression of VEGF

Since VEGF is known to promote vascular leakage, we wondered whether dexamethasone treatment altered this growth factor in our experimental model of VILI. Only after HV_T-ventilation, VEGF protein expression was significantly enhanced in comparison with NVC (figure 4). In both ventilation groups though, a significant downregulation in VEGF protein levels was noticed after dexamethasone administration.

Effect of dexamethasone treatment on ventilator-induced lung injury

To evaluate the effects of dexamethasone treatment on lung injury, we determined total protein levels in BALf, wet-to-dry ratios of lung tissues and PaO_2/FiO_2 ratios in blood samples. Figure 5a illustrates that BALf protein levels were higher in both LV_T and HV_T-ventilated mice compared to NVC, although the increase in the LV_T-ventilated group did not reach statistical significance ($p=0.052$). Wet-to-dry ratios of pulmonary tissue were increased after both ventilation strategies (figure 5b). Furthermore, HV_T-ventilation markedly decreased the PaO_2/FiO_2 ratio as compared to LV_T-ventilation (figure 5c). Treatment with dexamethasone did not restore any of these parameters of VILI (figure 5). In addition, we observed that

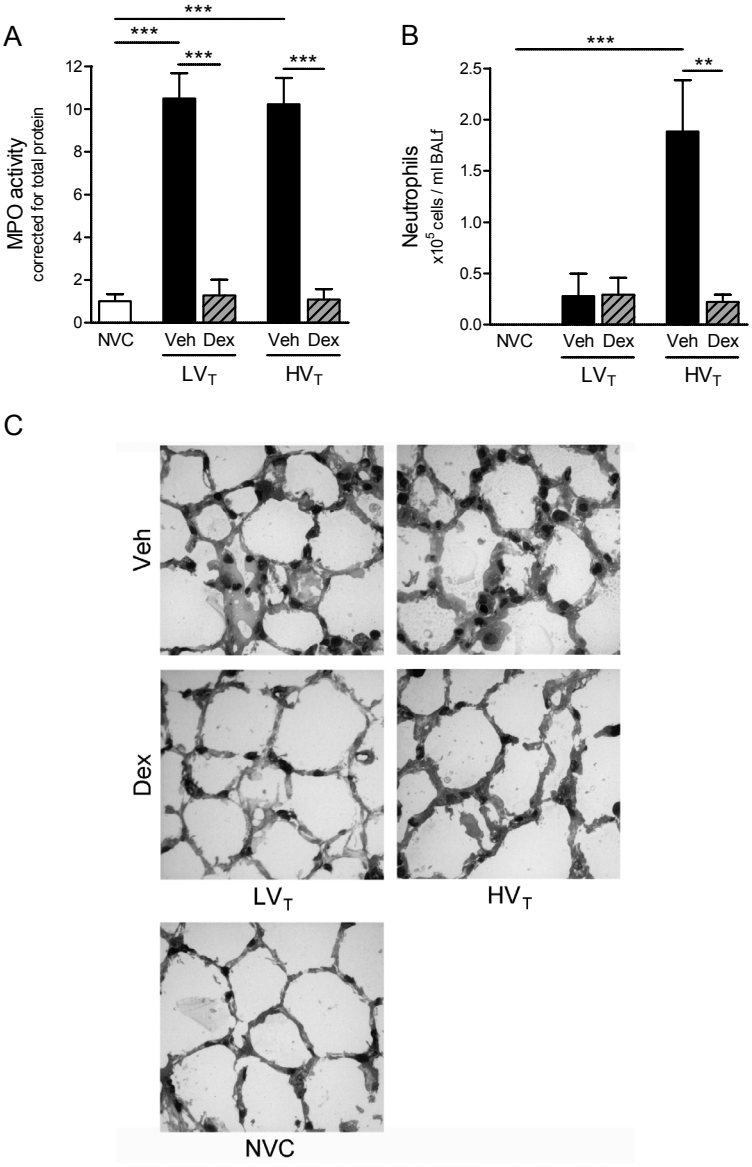

Figure 3: Dexamethasone treatment decreases infiltration of granulocytes. (See color figure on pg. 197.)

A: In total lung homogenates, myeloperoxidase (MPO) activity was determined as a measure of granulocyte infiltration. Levels were normalized for total protein concentrations. **B:** On bronchoalveolar lavage fluid (BALf) cytospin preparations, differential cell counts were done to determine neutrophil exudation into the alveolar space. Data are expressed as mean ± SEM, and depicted relative to NVC. A/B: NVC n=8/10, LV_T Veh n=16/6, LV_T Dex n=6/8, HV_T Veh n=12/17, HV_T Dex n=6/8. ** p<0.01, *** p<0.001. **C:** Lung sections were stained with hematoxylin-eosin (H&E) to analyze lung architecture and presence of granulocytes in pulmonary tissue. Magnification ×500.

NVC = non-ventilated controls; LV_T, HV_T = mechanically ventilated with low or high tidal volumes; Veh, Dex = intravenously treated with either vehicle (sterile saline) or dexamethasone.

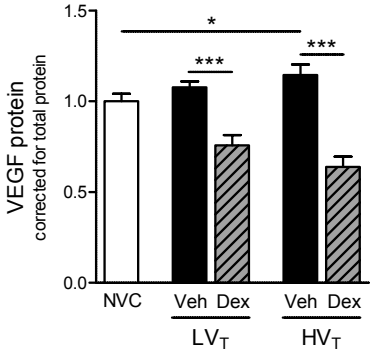

Figure 4: Dexamethasone treatment decreases protein expression of VEGF.
In total lung homogenates, protein expression of vascular endothelial growth factor (VEGF) was determined by multiplex cytokine analysis. Data are expressed as mean ± SEM, and depicted relative to NVC. NVC n=8, LV_T Veh n=9, LV_T Dex n=6, HV_T Veh n=9, HV_T Dex n=6. * $p<0.05$, *** $p<0.001$.
NVC = non-ventilated controls; LV_T, HV_T = mechanically ventilated with low or high tidal volumes; Veh, Dex = intravenously treated with either vehicle (sterile saline) or dexamethasone.

dexamethasone treatment in the HV_T-ventilation group was even aggravating the ventilator-induced effects on BALf total protein level and pulmonary wet-to-dry ratio, although the wet-to-dry ratio did not reach statistical significance (p=0.071, figure 5b).

DISCUSSION

The present study demonstrates that treatment with dexamethasone completely abolished expression of inflammatory mediators (KC, MIP-2, MCP-1, IL-1β, IL-6 and E-selectin), influx of granulocytes and expression of VEGF in lungs of LV_T and HV_T-ventilated mice. Moreover, we observed that dexamethasone administration preserved lung architecture during mechanical ventilation. Although dexamethasone was capable of inhibiting pulmonary inflammation and VEGF expression, it did not prevent the increase in BALf protein levels and pulmonary wet-to-dry ratios (i.e. vascular leakage) in both ventilation groups and the reduction in PaO_2/FiO_2 ratios in the HV_T-ventilation group. Interestingly, dexamethasone even aggravated vascular leakage induced by HV_T-ventilation.

Previous studies have reported that the presence of activated granulocytes in pulmonary tissue evokes oxidative stress and protease activity, thereby damaging epithelial-endothelial barriers and impairing gas exchange [7-9]. In our experimental model of VILI, both LV_T and HV_T-ventilation led to enhanced chemokine, cytokine and adhesion molecule expression accompanied by significant granulocyte infiltration in comparison with non-ventilated control animals. Moreover, we showed that BALf neutrophil numbers and inflammatory mediator expression were aggravated in lungs of HV_T-ventilated mice as compared to LV_T-ventilated

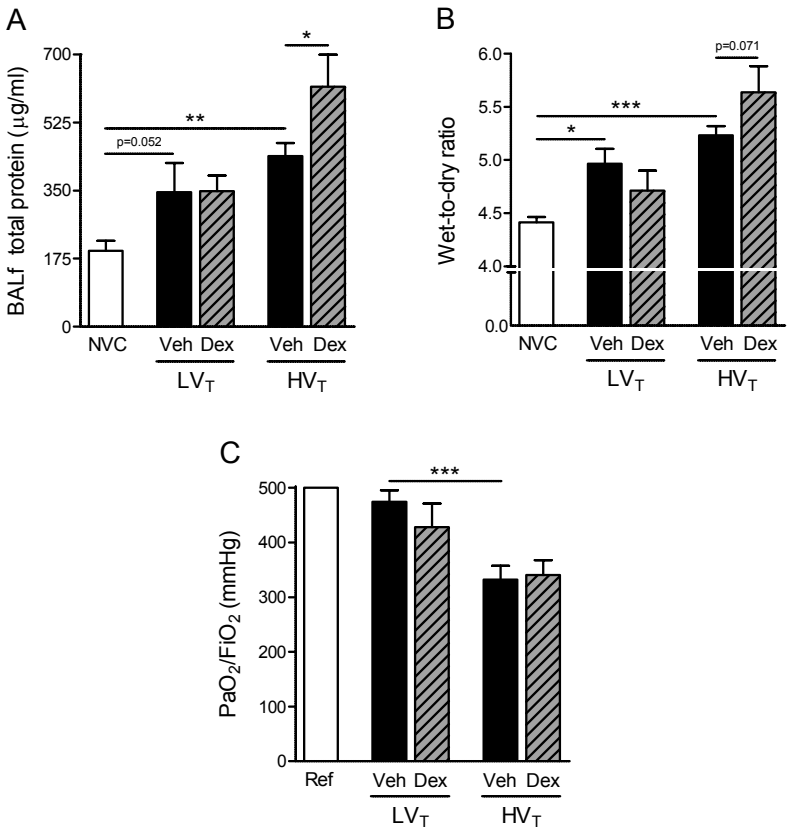

Figure 5: Dexamethasone treatment does not prevent ventilator-induced lung injury.
A: Alveolar-capillary permeability is represented by total protein levels in bronchoalveolar lavage fluid (BALf). **B:** Pulmonary edema is represented by wet-to-dry ratios of lung tissue. **C:** Oxygenation is represented by the ratio of partial pressure arterial oxygen and fraction inspired oxygen (PaO_2/FiO_2). Data are expressed as mean ± SEM. A-B: NVC n=12, LV_T Veh n=9-10, LV_T Dex n=10, HV_T Veh n=10, HV_T Dex n=10; C: LV_T Veh n=22, LV_T Dex n=11, HV_T Veh n=18, HV_T Dex n=11. * p<0.05, ** p<0.01, *** p<0.001.
Ref = reference bar (PaO_2/FiO_2 ratio from mice with non-injured lungs); NVC = non-ventilated controls; LV_T, HV_T = mechanically ventilated with low or high tidal volumes; Veh, Dex = intravenously treated with either vehicle (sterile saline) or dexamethasone.

mice. Since deteriorated PaO_2/FiO_2 ratios were only observed after HV_T-ventilation, it may well be that ventilator-induced lung inflammation ultimately leads to impaired gas exchange.

It has been hypothesized before that an inflammatory response in the lung may precede pulmonary injury in the pathogenesis of VILI [4-6]. Already in 1987, Kawano et al. described the importance of granulocyte-mediated tissue injury in the pathogenesis of VILI [21]. These authors showed that depletion of granulocytes improved gas exchange, vascular leakage and hyaline membrane formation in rabbits exposed to repetitive lung lavage and high-frequency oscillatory ventilation. Here we show that dexamethasone administration reduced

granulocyte infiltration into lungs of LV_T and HV_T-ventilated mice, which is in agreement with previous findings [13]. In addition, we observed that dexamethasone completely abolished mRNA and protein expression of inflammatory mediators implying that dexamethasone is capable of preventing lung inflammation induced by LV_T or HV_T-ventilation.

Apart from significant downregulation of pulmonary inflammation, we also found that dexamethasone treatment reduced expression of VEGF which is known to initiate vascular leakage in various experimental models of lung injury, including VILI [22-24]. Nonetheless, treatment with dexamethasone did not prevent the impairment in alveolar-capillary barrier function in LV_T and HV_T-ventilated mice and gas exchange in HV_T-ventilated mice. These data are in apparent contrast with earlier research showing protective effects of glucocorticoid treatment on lung injury [13,18]. In their rat model of VILI, Nin et al. demonstrated that dexamethasone treatment restored pulmonary injury after 75 minutes of mechanical ventilation as indicated by the improved PaO_2/FiO_2 ratio [18]. Furthermore, Ohta et al. observed a marked leftward shifting of the pressure-volume (P-V) curve of rats ventilated for 40 minutes and administered with methylprednisolone [13]. Yet, deterioration of the P-V curve was still evident regardless of significant reduction in granulocyte infiltration. Ohta et al. explained this discrepancy by the effects of mechanical stretch on lung tissue, such as stress failure of pulmonary capillaries, which may contribute to lung injury to a great extent [25]. Consequently, they proposed that it may be difficult to completely prevent the stretch-induced effects on lung injury merely by treatment with anti-inflammatory agents [13]. Since mice were exposed to 5 hours of mechanical ventilation in the present study, it is tempting to speculate that the progressive lung injury induced by prolonged mechanical stretch may not be influenced by the anti-inflammatory actions of dexamethasone anymore.

Although the use of glucocorticoids has shown promising results in other experimental models of VILI [13,18], the efficacy of glucocorticoids in treating ALI/ARDS in critically ill patients is still under debate [15-17]. Clinically, there is some support for our current experimental data. In a randomized controlled trial, Weigelt et al. observed that more ventilated, critically ill patients were diagnosed with ARDS after administration with methylprednisolone as compared to placebo (64% versus 33%, respectively) [26]. Enhanced incidence of ARDS was also described in another clinical trial where glucocorticoid therapy was started within 2 hours after the onset of sepsis [27]. Furthermore, these authors showed that the 14-day mortality rate was significantly higher in patients treated with methylprednisolone. So, randomized controlled trials failed to demonstrate protective effects of early corticosteroid treatment in patients at risk for ALI/ARDS. In line with these clinical observations, we show that dexamethasone treatment in mice without pre-existing lung injury did not prevent vascular leakage induced by LV_T or HV_T-ventilation and impaired gas exchange induced by HV_T-ventilation. Intriguingly, we observed that alveolar-capillary permeability and pulmonary edema even deteriorated in HV_T-ventilated mice. One explanation for our findings may be that LV_T and HV_T-ventilated mice were exposed to relatively high oxygen levels (FiO_2 of 0.5,

moderate hyperoxia). It has been reported that treatment with dexamethasone increased extravascular lung water in hyperoxia-exposed rats causing a shift in the onset of hyperoxic lung injury to an earlier time point [28]. The moderate hyperoxia associated with our ventilation strategies may therefore counterbalance the positive effects of dexamethasone on pulmonary injury so that the overall effect is neutral or even deleterious. Another explanation may be that the anti-inflammatory action of dexamethasone will also markedly reduce the growth-promoting effects of inflammatory mediators thereby suppressing potential repair mechanisms [29].

Similar to our data, Wolthuis et al. showed that the TNF-α inhibitor Etanercept reduced inflammation and coagulation in lungs of ventilated mice without affecting the end points of VILI, i.e. alveolar-capillary permeability and pulmonary edema [30]. Thus, downregulation of ventilator-induced lung inflammation by anti-inflammatory agents does not necessarily lead to improvement of lung injury as well. It should be noted, though, that healthy mice were used in these studies. Therefore, it may well be that anti-inflammatory agents are beneficial in individuals with pre-existing lung inflammation. The fact that glucocorticoid therapy showed protective effects when started in patients with established ARDS [31,32] supports this hypothesis.

Taken together, the present study shows that dexamethasone administration at initiation of mechanical ventilation prevents various aspects of VILI including inflammatory mediator release, granulocyte infiltration and VEGF expression. Nonetheless, this drug was not capable of preventing the more crude parameters of VILI such as alveolar-capillary permeability, pulmonary edema and impaired gas exchange. On the basis of our current data we propose that lung injury induced by prolonged mechanical stretch may not be responsive to dexamethasone treatment.

CONCLUSIONS

Although dexamethasone treatment inhibits lung inflammation and VEGF expression, it does not protect against vascular leakage induced by LV_T or HV_T-ventilation and impaired gas exchange induced by HV_T-ventilation.

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CHAPTER

5

Angiopoietin-1 treatment reduces inflammation but does not prevent ventilator-induced lung injury

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Submitted

ABSTRACT

Background: Loss of integrity of the epithelial and endothelial barriers is thought to be a prominent feature of ventilator-induced lung injury (VILI). Based on its function in vascular integrity, we hypothesize that the angiopoietin (Ang)-Tie2 system plays a role in the development of VILI. The present study was designed to examine the effects of mechanical ventilation on the Ang-Tie2 system in lung tissue. Moreover, we evaluated whether treatment with Ang-1, a Tie2 receptor agonist, protects against inflammation, vascular leakage and impaired gas exchange induced by mechanical ventilation.

Methods: Mice were anesthetized, tracheotomized and mechanically ventilated for 5 hours with either an inspiratory pressure of 10 cmH_2O ("low" tidal volume ~7.5 ml/kg; LV_T) or 18 cmH_2O ("high" tidal volume ~15 ml/kg; HV_T). At initiation of HV_T-ventilation, recombinant human Ang-1 was administered intravenously (1 or 4 μg per animal). Non-ventilated mice served as controls.

Results: HV_T-ventilation influenced the Ang-Tie2 system in lungs of healthy mice since Ang-1, Ang-2 and Tie2 mRNA were decreased. Treatment with Ang-1 increased Akt-phosphorylation indicating Tie2 signaling. Ang-1 treatment reduced infiltration of granulocytes and expression of keratinocyte-derived chemokine (KC), macrophage inflammatory protein (MIP)-2, monocyte chemotactic protein (MCP)-1 and interleukin (IL)-1β caused by HV_T-ventilation. Importantly, Ang-1 treatment did not prevent vascular leakage and impaired gas exchange in HV_T-ventilated mice despite inhibition of inflammation, vascular endothelial growth factor (VEGF) and Ang-2 expression.

Conclusions: Ang-1 treatment downregulates pulmonary inflammation, VEGF and Ang-2 expression but does not protect against vascular leakage and impaired gas exchange induced by HV_T-ventilation.

INTRODUCTION

Mechanical ventilation is an important life-saving procedure. However, the procedure itself may induce or aggravate damage to lung tissue, so-called ventilator-induced lung injury (VILI) [1,2]. VILI is characterized by inflammation, enhanced alveolar-capillary membrane permeability, accumulation of protein-rich pulmonary edema and ultimately impaired gas exchange [3]. Various animal models have been used to obtain further insight into the mechanisms underlying VILI. Already in the 1980s, investigators showed that mechanical ventilation and the subsequent mechanical (over)stretch of lung tissue induces damage to the epithelial-endothelial barrier leading to impaired oxygenation [4-6]. In addition, it has been described that the mechanical forces associated with mechanical ventilation provoke an inflammatory response in the lung (biotrauma) [7,8].

Loss of integrity of epithelial and endothelial cell monolayers has been suggested to play an important role in the ventilator-induced disruption of the alveolar-capillary barrier [9]. One of the crucial systems regulating vascular cell integrity is the angiopoietin (Ang)-Tie2 system [10]. Clarifying the role of the Ang-Tie2 system in the development of lung injury has therefore become a topic of great interest [11]. It has been recognized that Ang-1 serves as a Tie2 receptor agonist by phosphorylating Tie2 on tyrosine residues. Ang-1–mediated Tie2 signaling is required to maintain cellular integrity and quiescence of the endothelial barrier [10]. The antagonist Ang-2 is known to downregulate Tie2 signaling, thereby preparing vascular endothelial cells for enhanced responsiveness to factors that cause destabilization of the endothelial barrier [12]. However, there is also conflicting evidence that Ang-2 may induce Tie2 activation in stressed endothelial cells [13].

In a murine model of endotoxin-induced acute lung injury (ALI), Karmpaliotis et al. described that vascular permeability and pulmonary edema were accompanied by enhanced vascular endothelial growth factor (VEGF) and reduced Ang-1 levels in lung tissue [14]. The same authors proposed that changes in the balance between VEGF (pro-leakage) and Ang-1 (anti-leakage) might contribute to the pathophysiology of ALI. Protective effects of Ang-1 treatment have been shown before in experimental models of endotoxin-induced ALI [15-18]. Mei et al. demonstrated that treatment with Ang-1 attenuated vascular leakage, granulocyte infiltration and pro-inflammatory cytokine expression in lungs of endotoxin-exposed mice [17]. Consequently, the Ang-Tie2 system has been proposed as a possible therapeutic target in pulmonary diseases like ALI and its most severe form, the acute respiratory distress syndrome (ARDS) [11,19].

Vascular leakage and pulmonary inflammation are important features of VILI. Therefore, we hypothesized that Ang-1–Tie2 signaling plays a (protective) role in the development of VILI. In an attempt to better reflect the human setting, we applied a relatively mild model of VILI using clinically relevant ventilator settings thereby preventing shock, metabolic acidosis and substantial damage to lung architecture [20]. The aim of present study was to investigate

the influence of mechanical ventilation on the Ang-Tie2 system in lungs of healthy adult mice. Furthermore, we examined whether treatment with Ang-1, a Tie2 receptor agonist, would protect ventilated mice against important hallmarks of VILI such as inflammation, vascular leakage and impaired gas exchange.

MATERIALS AND METHODS

Animals

Experiments were performed in accordance with international guide lines and approved by the animal care and use committees of the University Medical Center Utrecht and the Academic Medical Center Amsterdam. Adult male C57Bl6 mice (n=145; Charles River, Maastricht, the Netherlands), weighing 20 to 24 grams, were randomly assigned to different experimental groups.

Study design

Mice (n=114) were mechanically ventilated for 5 hours as described previously [20]; pressure-controlled, fractional inspired oxygen concentration (FiO_2) of 0.5, inspiration-to-expiration ratio of 1:1 and positive end-expiratory pressure of 2 cmH_2O. Six mice were simultaneously ventilated with either an inspiratory pressure of 10 cmH_2O (resulting in "low" tidal volume (V_T) ~7.5 ml/kg; LV_T) or 18 cmH_2O (resulting in "high" V_T ~15 ml/kg; HV_T). Respiratory rate was set at 100 and 50 breaths/min, respectively. Body temperature was kept constant between 36.5 and 37.5°C. Non-ventilated mice (n=31) served as controls (non-ventilated controls, NVC).

Ang-1 treatment

At initiation of HV_T-ventilation, recombinant human Ang-1 (carrier free; R&D systems, Minneapolis, MN) was intravenously administered (either 1 or 4 µg per animal). Control HV_T-ventilated mice received the same volume of sterile saline (vehicle) intravenously.

Hemodynamics and blood gas analysis

After 0, 2.5 and 5 hours, systolic blood pressure and heart rate were non-invasively monitored using a tail-cuff system (ADInstruments, Spenbach, Germany). After 5 hours, arterial blood was taken from the carotid artery for blood gas analysis (Rapidlab 865; Bayer, Mijdrecht, the Netherlands).

Bronchoalveolar lavage

The right lung was lavaged by instilling 3x 0.5 ml sterile saline. Differential counts were done on cytospin preparations stained with Giemsa (Diff-Quick; Dade Behring AG, Düdingen, Switzerland). Cell-free supernatant was used to measure total protein (BCA protein-assay; Pierce Biotechnology, Rockford, IL) with BSA as standard.

Wet-to-dry ratio

The left lung was weighed, dried for 3 days (65°C) and weighed again.

Histopathology and immunohistochemistry

The left lung was filled with Tissue-Tek® (Sakura Finetek, Zoeterwoude, the Netherlands), snap frozen and cut to 5 µm cryosections using a cryostat. To assess pulmonary histopathology, sections were stained with hematoxylin-eosin (H&E; Klinipath, Duiven, the Netherlands). To assess Tie2 localization, sections were stained with fluorescent antibody recognizing Tie2 (Tek4; eBioscience, San Diego, CA) or isotype control antibody (IgG1-biotin).

Tissue homogenate preparation

Lung tissue was pulverized using a liquid nitrogen-cooled mortar and pestle, and divided in several fractions allowing us to perform multiple analyses (as described below).

Real-time RT-PCR analysis

PCR was performed as described previously [21]. Primer sequences: Ang-1, forward CTACCAACAACAACAgCATCC, reverse CTCCCTTTAgCAAAACACCTTC; Ang-2, forward CTgTgCggAAATCTTCAAgTC, reverse TgCCATCTTCTCggTgTT; Tie2, forward gTgTAgTggACCAgAAgg, reverse CTTgAgAgCAgAggCATC. Data were normalized for expression of internal controls, i.e. the average value of β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPdH).

Western blotting

Western blotting was performed as described previously [22]. Antibodies recognized phospho (p)-Akt (Ser473; Cell Signaling, Danvers, MA), Ang-1 or Ang-2 (both Alpha Diagnostic, San Antonio, TX). To control for equal loading, membranes were stripped when necessary and reprobed with an antibody recognizing total Akt (Akt1/PKBα; Sigma-Aldrich, Steinheim, Germany).

ELISA

Tie2 protein was measured by ELISA according to manufacturer's instructions (R&D).

Myeloperoxidase (MPO) assay

MPO activity was determined as described previously [21].

Multiplex cytokine analysis

125 µg protein was analyzed for keratinocyte-derived chemokine (KC), macrophage inflammatory protein (MIP)-2, monocyte chemotactic protein (MCP)-1, interleukin (IL)-1β, IL-6, IL-10 and VEGF by multiplex cytokine assay using a Luminex analyzer (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's instructions (multiplex mouse cytokine, R&D).

Statistical analysis

Data are expressed as mean ± SEM. Oxygenation variables (LV_T versus HV_T) and Ang-1, Ang-2, Tie2 protein (NVC versus HV_T) were analyzed by independent T-test. Other parameters were analyzed by one-way ANOVA with least significant difference (LSD) post-hoc test. P-values less than 0.05 were considered statistically significant.

RESULTS

Stability of the murine model of VILI

All mice survived 5 hours of LV_T and HV_T-ventilation after which they were sacrificed. Analysis of systolic blood pressure and heart rate revealed stable conditions of ventilated mice throughout the experiment, with no differences between ventilation strategies (table 1).

Table 2 illustrates that arterial oxygen tension (PaO_2) was reduced in HV_T-ventilated mice when compared to LV_T-ventilated mice. Carbon dioxide tension ($PaCO_2$), pH and base excess (BE) remained within the physiological range during both LV_T and HV_T-ventilation.

Effect of mechanical ventilation on alveolar-capillary permeability, pulmonary edema formation and gas exchange

First, we examined whether LV_T and HV_T-ventilation caused lung injury in mice without pre-existing lung injury. Total protein levels in bronchoalveolar lavage fluid (BALf), wet-to-dry

Table 1. Hemodynamic characteristics over 5 hours of mechanical ventilation

	LV_T	HV_T
$HR_{t=0\ hr}$	370 ± 11	387 ± 17
$HR_{t=2,5\ hr}$	382 ± 11	336 ± 13
$HR_{t=5\ hr}$	403 ± 12	351 ± 9
$BP_{t=0\ hr}$	104 ± 4	97 ± 6
$BP_{t=2,5\ hr}$	76 ± 6	72 ± 4
$BP_{t=5\ hr}$	73 ± 5	74 ± 5

Data are expressed as mean ± SEM (LV_T n=11-12, HV_T n=12). LV_T, HV_T = mechanically ventilated with low or high tidal volumes; HR = heart rate in beats/minute; BP = systolic blood pressure in mmHg.

Table 2. Arterial blood gas analysis after 5 hours of mechanical ventilation

	LV_T	HV_T
PaO_2	231,6 ± 15,8	165,8 ± 12,8*
$PaCO_2$	32,4 ± 3,4	35,1 ± 2,5
pH	7,51 ± 0,03	7,50 ± 0,02
BE	2,08 ± 0,78	3,54± 0,91

Data are expressed as mean ± SEM (LV_T n=16, HV_T n=18). * $p<0.01$ versus LV_T. LV_T, HV_T = mechanically ventilated with low or high tidal volumes; PaO_2 = partial pressure of arterial oxygen in mmHg; $PaCO_2$ = partial pressure of arterial carbon dioxide in mmHg; BE = base excess in mmol/l.

ratios of pulmonary tissue and PaO_2/FiO_2 ratio in blood samples were analyzed to evaluate the effects of mechanical ventilation on alveolar-capillary permeability, pulmonary edema formation and gas exchange respectively. We found that both ventilation strategies markedly enhanced BALf protein levels and pulmonary wet-to-dry ratios in comparison with NVC (figures 1a and b). Furthermore, PaO_2/FiO_2 ratios were significantly decreased when comparing HV_T-ventilated mice with LV_T-ventilated mice (figure 1c).

Effect of mechanical ventilation on the Ang-Tie2 system

To assess whether 5 hours of mechanical ventilation influenced the Ang-Tie2 system, we determined Ang-1, Ang-2 and Tie2 expression in total lung homogenates. We found that only HV_T-ventilation caused a decrease in Ang-1 and Ang-2 mRNA compared to NVC (figures 2a and b). Both ventilation strategies reduced Tie2 mRNA (figure 2c). Since ventilator-induced effects on the Ang-Tie2 system were most pronounced after HV_T-ventilation, we continued our investigations by focusing on this specific group.

To determine if ventilator-induced downregulation of Ang-1, Ang-2 and Tie2 mRNA also resulted in a reduction of Ang-1, Ang-2 and Tie2 protein, we measured protein expression of these mediators in total lung homogenates. Decreased Ang-1 and Ang-2 protein levels were observed after 5 hours of HV_T-ventilation, although these differences did not reach statistical

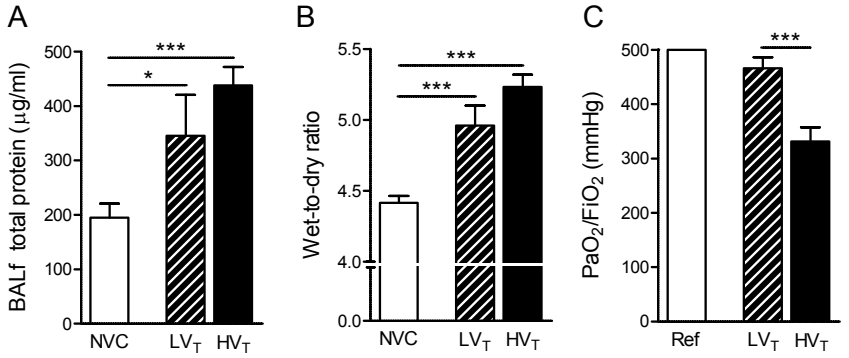

Figure 1: Mechanical ventilation affects alveolar-capillary permeability, pulmonary edema formation and gas exchange.

A: Alveolar-capillary permeability is represented by total protein levels in bronchoalveolar lavage fluid (BALf). **B:** Pulmonary edema is represented by wet-to-dry ratios of lung tissue. **C:** Oxygenation is represented by the ratio of partial pressure arterial oxygen and fraction inspired oxygen (PaO_2/FiO_2). Data are expressed as mean ± SEM. A/B: NVC n=12, LV_T n=9/10, HV_T n=10; C: LV_T n=21, HV_T n=18. * $p<0.05$, *** $p<0.001$.

Ref = reference bar (PaO_2/FiO_2 ratio from mice with non-injured lungs); NVC = non-ventilated controls; LV_T, HV_T = mechanically ventilated with low or high tidal volumes.

significance (p=0.084 and p=0.058 respectively, figures 2d and e). In addition, HV_T-ventilation did not lead to downregulation of Tie2 protein expression (figure 2f). To evaluate the localization of Tie2 in pulmonary tissue, Tie2 protein was visualized by immunofluorescent staining on lung sections of NVC. As illustrated in figure 2g, Tie2 was primarily detected in larger arterioles and alveolar-capillary membranes (isotype control was negative).

Effect of Ang-1 treatment on phospho-Akt (p-Akt), Ang-2 and VEGF expression

Mice were treated with sterile saline (vehicle), 1 µg Ang-1 or 4 µg Ang-1 per animal at initiation of HV_T-ventilation and subsequently ventilated for 5 hours. To explore whether Ang-1 administration induces Tie2 signaling in lungs of HV_T-ventilated mice, we determined the level of p-Akt protein. Akt phosphorylation is known to be induced by Tie2 signaling, and thus, increased levels of p-Akt may serve as indirect evidence of Tie2 receptor activation. HV_T-ventilation induced phosphorylation of Akt compared to NVC and Ang-1 treatment enhanced the level of p-Akt even further (figure 3). This increase was most distinct when using a dose of 4 µg Ang-1 per animal.

We also studied if Ang-1 treatment altered the expression of factors promoting vascular leakage like Ang-2 and VEGF. On mRNA level, Ang-2 expression was downregulated in lungs of HV_T-ventilated mice treated with 1 or 4 µg Ang-1 (figure 4a). At this time point, no effect of Ang-1 administration was observed on Ang-2 protein expression (figure 4b). Compared

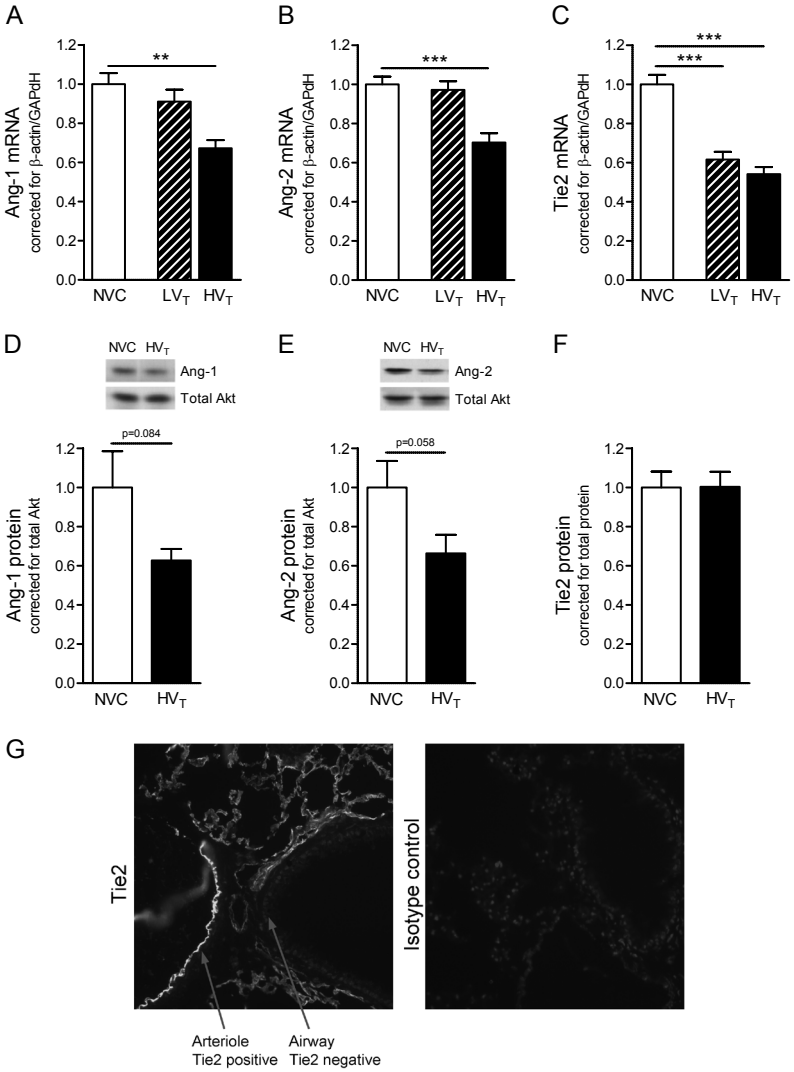

Figure 2: Mechanical ventilation affects the angiopoietin (Ang)-Tie2 system. (See color figure on pg. 198.)

A-C: In total lung homogenates, mRNA expression of Ang-1, Ang-2 and Tie2 was determined by real-time RT-PCR. Levels were normalized for expression of internal controls, i.e. the average value of β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPdH). **D-E:** In total lung homogenates, protein expression of Ang-1 and Ang-2 was determined by Western blotting. Membranes were reprobed with antibody recognizing total Akt (Akt1/PKBα) to control for equal loading. No group differences in total Akt were found. Inset: representative Western blot depicting immunodetectable Ang-1 and Ang-2. **F:** In total lung homogenates, protein expression of Tie2 was determined by ELISA. Levels were normalized for total protein concentrations. Data are expressed as mean ± SEM, and depicted relative to NVC. A-C: NVC n=6, LV$_T$ n=16, HV$_T$ n=12; D/E: NVC n=9/10, HV$_T$ n=8/10; F: NVC n=6, HV$_T$ n=6. ** p<0.01, *** p<0.001. **G:** Lung sections of NVC were stained with fluorescent antibody recognizing Tie2 to visualize the presence of Tie2 on pulmonary cells (isotype control was negative). Magnification ×200.

NVC = non-ventilated controls; LV$_T$, HV$_T$ = mechanically ventilated with low or high tidal volumes.

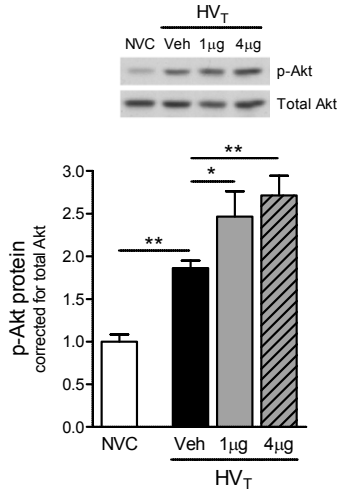

Figure 3: Angiopoietin (Ang)-1 treatment induces Tie2 signaling.
In total lung homogenates, protein expression of phospho (p)-Akt was determined by Western blotting as an indirect measure of Tie2 signaling. Membranes were stripped and reprobed with antibody recognizing total Akt (Akt1/PKBα) to control for equal loading. No group differences in total Akt were found. Inset: representative Western blot depicting immunodetectable p-Akt. Data are expressed as mean ± SEM, and depicted relative to NVC. NVC n=10, HV_T Veh n=10, HV_T 1μg n=8, HV_T 4μg n=10. * p<0.05, ** p<0.01. NVC = non-ventilated controls; HV_T = ventilated with high tidal volumes; Veh, 1μg, 4μg = intravenously treated with either vehicle (sterile saline), Ang-1 (1μg per animal), or Ang-1 (4μg per animal).

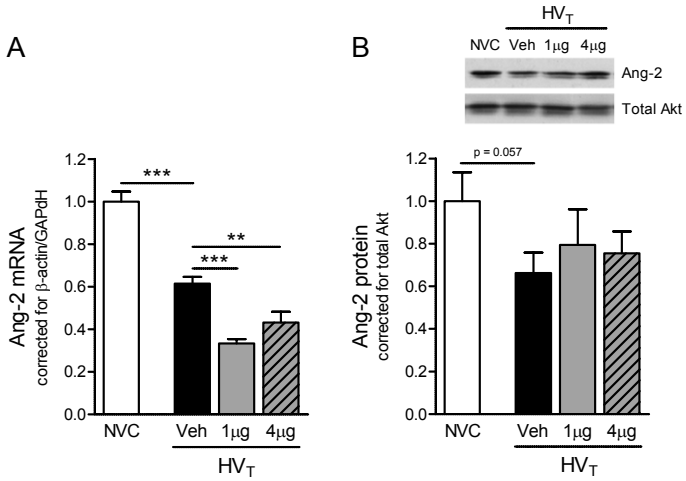

Figure 4: Angiopoietin (Ang)-1 treatment reduces Ang-2 mRNA expression.
A: In total lung homogenates, mRNA expression of Ang-2 was determined by real-time RT-PCR. Levels were normalized for expression of internal controls, i.e. the average value of β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPdH). **B:** In total lung homogenates, protein expression of Ang-2 was determined by Western blotting. Membranes were reprobed with antibody recognizing total Akt (Akt1/PKBα) to control for equal loading. No group differences in total Akt were found. Inset: representative Western blot depicting immunodetectable Ang-2. Data are expressed as mean ± SEM, and depicted relative to NVC. A/B: NVC n=10, HV_T Veh n=16/10, HV_T 1μg n=7/8, HV_T 4μg n=10. ** p<0.01, *** p<0.001.

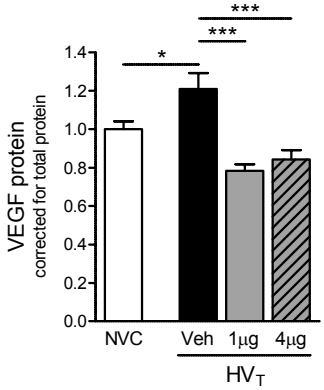

Figure 5: Angiopoietin (Ang)-1 treatment reduces VEGF protein expression.
In total lung homogenates, protein expression of vascular endothelial growth factor (VEGF) was determined by multiplex cytokine analysis. Data are expressed as mean ± SEM, and depicted relative to NVC. NVC n=8, HV_T Veh n=10, HV_T 1µg n=8, HV_T 4µg n=8. * $p<0.05$, *** $p<0.001$.
NVC = non-ventilated controls; HV_T = mechanically ventilated with high tidal volumes; Veh, 1µg, 4µg = intravenously treated with either vehicle (sterile saline), Ang-1 (1µg per animal), or Ang-1 (4µg per animal).

to NVC, VEGF protein expression was increased in the vehicle-treated HV_T-group (figure 5). Both doses of Ang-1 completely abolished the increase in VEGF protein in response to HV_T-ventilation.

Effect of Ang-1 treatment on ventilator-induced granulocyte infiltration into pulmonary tissue and granulocyte exudation into the alveolar space

To determine the effect of Ang-1 treatment on inflammatory activity in the lung, we first quantified granulocyte infiltration by measuring MPO activity in total lung homogenates and by counting neutrophils on BALf cytospin preparations. Compared to NVC, MPO activity and neutrophil numbers were markedly higher in lungs of HV_T-ventilated mice (figures 6a and b). Administration of either 1 or 4 µg Ang-1 reduced granulocyte influx after HV_T-ventilation (figure 6a). Supporting the MPO data, neutrophil numbers on BALf cytospin preparations were diminished in HV_T-ventilated mice treated with 1µg Ang-1 as compared to HV_T-ventilated mice treated with vehicle (figure 6b). In addition, we stained lung sections for H&E to visualize granulocytes in pulmonary tissue. Figure 6c illustrates that both doses of Ang-1 prevented the appearance of granulocytes in lungs of HV_T-ventilated mice, confirming the quantitative measures for infiltrating granulocytes.

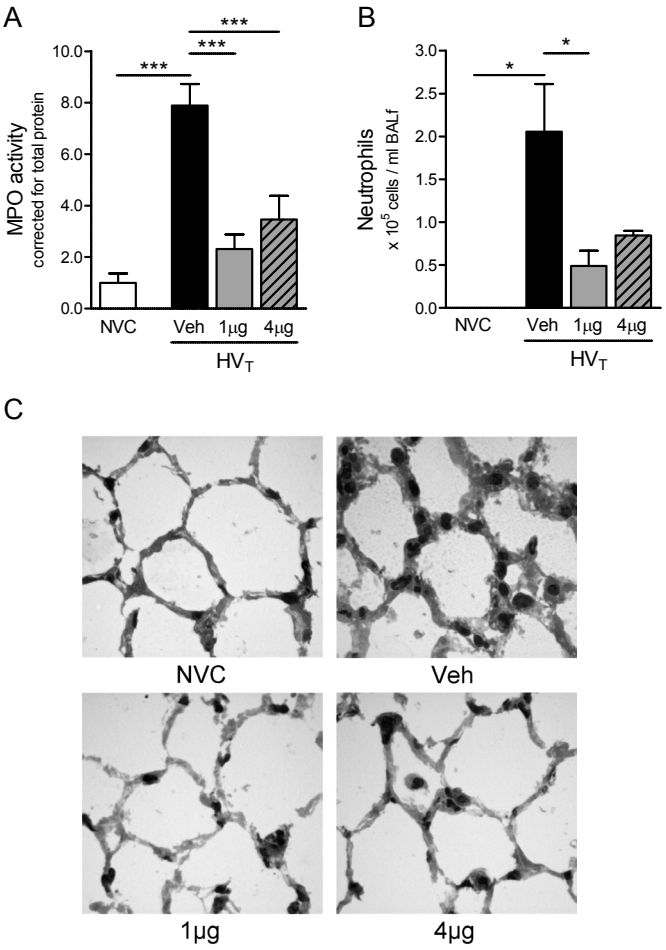

Figure 6: Angiopoietin (Ang)-1 treatment reduces granulocyte infiltration. (See color figure on pg. 199.)

A: In total lung homogenates, myeloperoxidase (MPO) activity was determined as a measure of granulocyte infiltration. Levels were normalized for total protein concentrations. **B:** On bronchoalveolar lavage fluid (BALf) cytospin preparations, differential cell counts were done to determine neutrophil exudation into the alveolar space. Data are expressed as mean ± SEM, and depicted relative to NVC. A/B: NVC n=8/5, HV_T Veh n=16/15, HV_T 1µg n=8/6, HV_T 4µg n=9/6. * $p<0.05$, *** $p<0.001$. **C:** Lung sections were stained with hematoxylin-eosin (H&E) to analyze the presence of granulocytes in pulmonary tissue. Magnification ×500.

NVC = non-ventilated controls; HV_T = mechanically ventilated with high tidal volumes; Veh, 1µg, 4µg = intravenously treated with either vehicle (sterile saline), Ang-1 (1µg per animal), or Ang-1 (4µg per animal).

Effect of Ang-1 treatment on ventilator-induced chemokine and cytokine expression

The effect of Ang-1 treatment on the levels of inflammatory mediators expressed by pulmonary tissue during HV_T-ventilation was determined as an additional measure of inflammatory activity in the lung. HV_T-ventilation induced protein expression of the chemokines KC, MIP-2 and MCP-1 in comparison with NVC (figures 7a to c). Consistent with the diminished granulocyte influx, treatment with either 1 or 4 µg Ang-1 reduced the upregulation of these chemokines after 5 hours of HV_T-ventilation. Moreover, higher levels of the pro-inflammatory cytokine IL-1β were found in pulmonary tissue of HV_T-ventilated mice (figure 7d). The elevated protein expression of IL-6 did not reach statistical significance (p=0.064, figure 7e). Although Ang-1 treatment decreased the expression of IL-1β protein compared to the HV_T-vehicle group, it did not influence the expression of IL-6 (figures 7d and e). The anti-inflammatory cytokine IL-10 was below detection level in all experimental groups.

Effect of Ang-1 treatment on ventilator-induced lung injury

Ang-1 treatment suppressed the inflammatory activity in the lung. To determine the consequences for lung injury, we evaluated whether the increase in vascular leakage and decrease in oxygenation during HV_T-ventilation could be restored by Ang-1 administration. As depicted in figures 8a and b, the increased BALf protein levels and pulmonary wet-to-dry ratios of HV_T-ventilated mice were not affected by treatment with either 1 or 4 µg Ang-1. Furthermore, both doses of Ang-1 did not prevent the reduction in PaO_2/FiO_2 ratio caused by HV_T-ventilation (figure 8c).

DISCUSSION

At the best of our knowledge this is the first report demonstrating that mechanical ventilation affects the Ang-Tie2 system in pulmonary tissue of healthy adult mice. Particularly in lungs of HV_T-ventilated mice, we observed marked changes in the Ang-Tie2 system. Compared to NVC, 5 hours of HV_T-ventilation resulted in downregulation of Ang-1, Ang-2 and Tie2 mRNA expression. In addition, Ang-1 and Ang-2 protein expression tended to decrease in HV_T-ventilated mice at this time point. The major finding of the present study is that treatment with Ang-1 affected only specific aspects of VILI. Ang-1 administration at initiation of ventilation diminished granulocyte infiltration, as well as chemokine (KC, MIP-2, MCP-1), cytokine (IL-1β), VEGF and Ang-2 expression in lungs of HV_T-ventilated mice. However, Ang-1 treatment did not prevent the increase in BALf total protein level, the increase in pulmonary wet-to-dry ratio and the reduction in PaO_2/FiO_2 ratio induced by HV_T-ventilation. This result

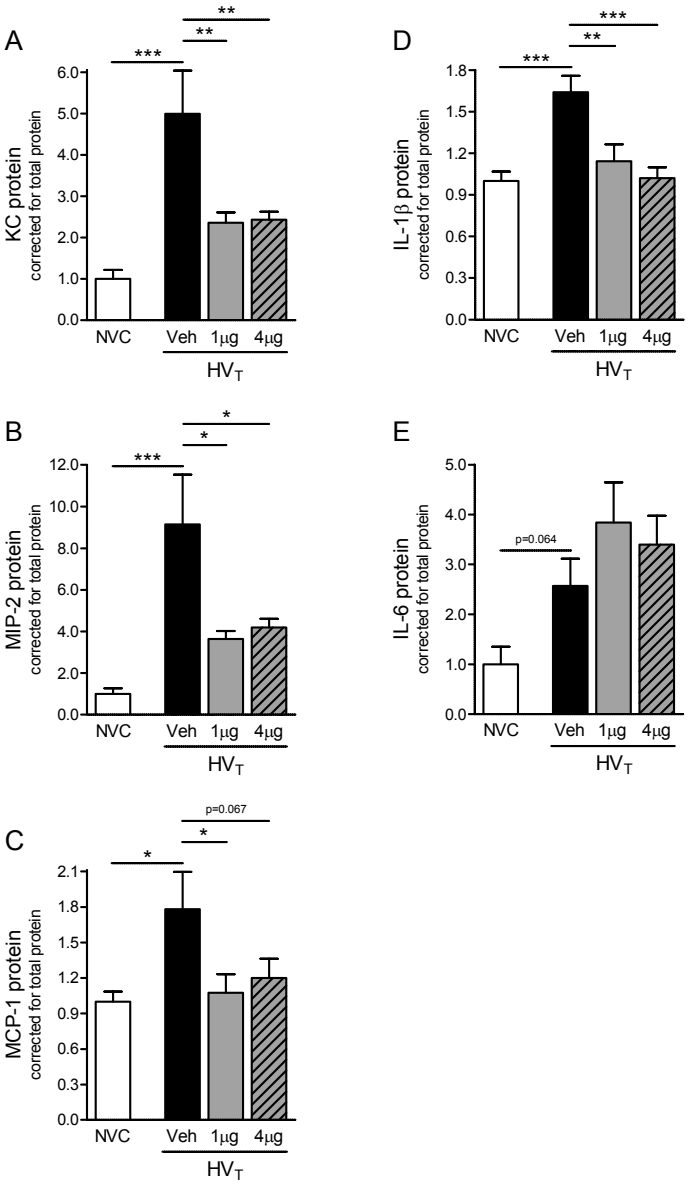

Figure 7: Angiopoietin (Ang)-1 treatment reduces chemokine and IL-1β protein expression.
A-E: In total lung homogenates, protein expression of the chemoattractants keratinocyte-derived chemokine (KC), macrophage inflammatory protein (MIP)-2, monocyte chemotactic protein (MCP)-1 and the pro-inflammatory cytokines interleukin (IL)-1β, IL-6 was determined by multiplex cytokine analysis. Data are expressed as mean ± SEM, and depicted relative to NVC. NVC n=8, HV_T Veh n=10, HV_T 1μg n=8, HV_T 4μg n=8. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.
NVC = non-ventilated controls; HV_T = mechanically ventilated with high tidal volumes; Veh, 1μg, 4μg = intravenously treated with either vehicle (sterile saline), Ang-1 (1μg per animal), or Ang-1 (4μg per animal).

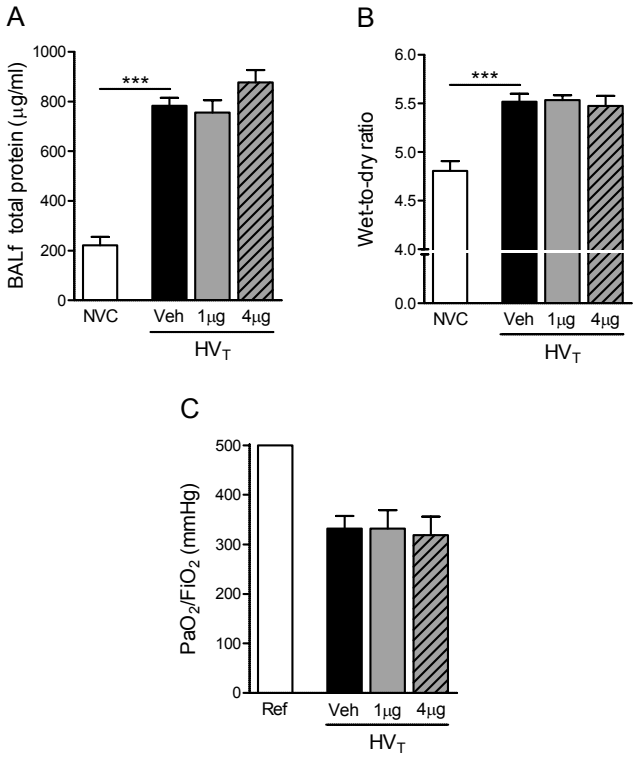

Figure 8: Angiopoietin (Ang)-1 treatment does not prevent alveolar-capillary permeability, pulmonary edema formation and impaired gas exchange.

A: Alveolar-capillary permeability is represented by total protein levels in bronchoalveolar lavage fluid (BALf). **B:** Pulmonary edema is represented by wet-to-dry ratios of lung tissue. **C:** Oxygenation is represented by the ratio of partial pressure arterial oxygen and fraction inspired oxygen (PaO_2/FiO_2). Data are expressed as mean ± SEM. A/B: NVC n=5/6, HV_T Veh n=15/14, HV_T 1µg n=6/8, HV_T 4µg n=5/8; C: HV_T Veh n=18, HV_T 1µg n=14, HV_T 4µg n=14. *** p<0.001.

Ref = reference bar (PaO_2/FiO_2 ratio from mice with non-injured lungs); NVC = non-ventilated controls; HV_T = mechanically ventilated with high tidal volumes; Veh, 1µg, 4µg = intravenously treated with either vehicle (sterile saline), Ang-1 (1µg per animal), or Ang-1 (4µg per animal).

was not only observed after administration of 1 µg of Ang-1 but also after administration of 4 µg of Ang-1. In view of these data, we would like to suggest that Ang-1 treatment does not prevent the aspects of VILI driven by mechanosensitive alterations in barrier properties [23] but will only regulate the pulmonary inflammation.

In experimental studies, mechanical ventilation has been described to induce destabilization of the alveolar-capillary barrier thereby leading to enhanced pulmonary permeability and edema formation [4-6]. Most models of VILI, however, applied very high inspiratory pressures or tidal volumes when compared to those used in the human setting [24-28]. To prevent shock and metabolic acidosis due to very high inspiratory pressures or tidal volumes, we

used ventilation strategies with more clinically relevant ventilator settings [20]. Even in this relatively mild model of VILI, we observed that mechanical ventilation caused a modest, but significant increase in BALf protein level and pulmonary wet-to-dry ratio.

The importance of the Ang-Tie2 system has been appreciated in the development of vascular leakage and inflammation in pulmonary diseases like ALI/ARDS [11]. In this respect, Karmpaliotis et al. proposed that changes in the balance between VEGF (pro-leakage) and Ang-1 (anti-leakage) might contribute to vascular leakage in their murine model of lipopolysaccharide (LPS)-induced ALI [14]. Here we demonstrate that HV_T-ventilation as such enhanced expression of VEGF protein, decreased expression of Ang-1, Ang-2 and Tie2 mRNA and tended to reduce expression of Ang-1 and Ang-2 protein compared to NVC. Since the mice were sacrificed after 5 hours of HV_T-ventilation, we could not evaluate whether the downregulation in mRNA expression was followed by a significant decrease in protein expression at a later time point. Even so, the present study suggests that alterations in the Ang-Tie2 system are involved in the pathogenesis of VILI.

Kim et al. and Papapetropoulos et al. have demonstrated that Ang-1–mediated Tie2 signaling causes Akt phosphorylation thereby protecting the endothelial cells from apoptotic cell death [29,30]. In our study, HV_T-ventilation itself caused increased p-Akt levels compared to NVC which is in agreement with previous reports [31,32]. Moreover, treatment with Ang-1 augmented p-Akt expression in lungs of HV_T-ventilated mice. (Ang-1–mediated) Akt phosphorylation has been shown to inactivate the forkhead transcription factor FKHR1 subsequently preventing Ang-2 expression and destabilization of the endothelial barrier [33]. This observation supports our finding that HV_T-ventilation as such reduced Ang-2 mRNA and that Ang-1 treatment downregulated the transcription of Ang-2 even further. The Ang-1–induced shift towards less Ang-2 production, thus reduced Tie2 antagonism, may protect against lung inflammation and injury during HV_T-ventilation.

In agreement with previous reports [26-28], we observed that ventilator-induced lung injury was accompanied by enhanced pro-inflammation. In LPS-challenged animals, treatment with Ang-1 has already been shown to decrease leukocyte trafficking by reducing chemotactic, adhesive and pro-inflammatory mediators [17]. Our study is the first to show that Ang-1 administration reduced infiltration of granulocytes and expression of the chemokines KC, MIP-2 and MCP-1 in an experimental model of VILI. Furthermore, we observed that administration of Ang-1 prevented the increase in the pro-inflammatory cytokine IL-1β in lungs of HV_T-ventilated mice. Our data may suggest that the role of IL-1β might be less important in the development of vascular leakage during HV_T-ventilation. Interestingly, IL-6 protein levels remained high in lungs of HV_T-ventilated mice despite Ang-1 treatment. In pulmonary inflammation, IL-1β is primarily produced by activated alveolar macrophages whereas IL-6 may be derived from a wide variety of pulmonary cell types [34]. Since Tie2 is expressed on endothelial cells, neutrophils and macrophages [35,36], our observations may imply that IL-6 is also derived from cells that do not express the Tie2 receptor and consequently do not respond to Ang-1.

The angiogenic growth factor VEGF has been shown to increase capillary permeability and edema formation in various experimental models of pulmonary injury, including VILI [14,37,38]. Thurston et al. described that vascular leakage induced by VEGF may be counteracted by Ang-1 [39,40]. In line with this notion, we observed that Ang-1 treatment completely abolished the increase in VEGF protein in lungs of HV_T-ventilated mice. Nonetheless, it should be noted that Ang-1 treatment did not prevent alveolar-capillary permeability, pulmonary edema (i.e. vascular leakage) and impaired gas exchange induced by HV_T-ventilation. These data are in apparent contrast with previously described protective effects of Ang-1 on vascular leakage in endotoxin-challenged animals [15-18] underlining that the pathways involved in endotoxin- and ventilator-induced lung injury are different. An explanation for this discrepancy might be that the enhanced inflammation is not the primary inducer of vascular leakage and impaired gas exchange during HV_T-ventilation, as is the case in the induction of lung injury by LPS. It has been demonstrated that ventilator-induced mechanical stretch may also lead to the destabilization of alveolar-epithelial and capillary-endothelial barriers thereby resulting in increased vascular permeability and pulmonary edema [4-6]. Ang-1 administration will probably influence the capillary-endothelial but not the alveolar-epithelial barrier since the Tie2 receptor is mainly expressed on endothelial cells. Thus, the possibility remains that Ang-1 treatment is not capable of restoring lung injury induced by HV_T-ventilation as it only modulates endothelial inflammation. The fact that Ang-1 prevents pulmonary vascular leakage in animals exposed to LPS, which induces a generalized inflammation primarily in the endothelial cells of the lung [41], supports this hypothesis.

Taken together, our data indicate that treatment with Ang-1 inhibits various aspects of VILI such as granulocyte infiltration, chemokine/cytokine and VEGF expression. However, Ang-1 treatment did not protect HV_T-ventilated mice against the more crude parameters of VILI (i.e. vascular leakage and impaired gas exchange). In this respect, it is of interest that the TNF-α inhibitor Etanercept diminished inflammation and coagulation in the lungs of ventilated mice without influencing alveolar-capillary permeability and pulmonary edema, which is in line with our present results [42]. We propose that Ang-1 should not be applied to combat the mechanosensitive aspects of ventilator-induced lung injury in critically ill patients. Nonetheless, treatment with Ang-1 may well be considered as an anti-inflammatory therapy when inflammation is the primary cause of lung injury.

CONCLUSIONS

Treatment with Ang-1 significantly reduces pulmonary inflammation, VEGF and Ang-2 expression but does not prevent vascular leakage and impaired oxygenation induced by HV_T-ventilation.

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CHAPTER

6

Activation of caspase-dependent and caspase-independent cell death pathways after lipopolysaccharide-induced lung injury in rats

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Submitted

ABSTRACT

Background: Acute lung injury (ALI) is characterized by lung inflammation and impaired gas exchange. Pulmonary cell death has been considered as an underlying mechanism in ALI. The aim of the present study was to delineate various cell death pathways during ALI.

Methods: Rats were intratracheally instilled with lipopolysaccharide (LPS) to induce lung injury. At 4, 12 and 24 hours after LPS instillation, activation of caspase-dependent and caspase-independent cell death pathways were analyzed in lung homogenates.

Results: LPS increased the level of cleaved caspase-3 at 12 hours with a further increase at 24 hours indicating that caspase-dependent apoptotic pathways were activated. From 12 hours after LPS-challenge, mitochondrial cytochrome *c* levels decreased and cleaved caspase-9 levels were upregulated suggesting a role for activation of the intrinsic apoptotic pathway. Cleaved caspase-8 and its product truncated Bid were increased at 4 hours after LPS and declined over time indicating that the extrinsic apoptotic pathway was activated early after LPS instillation. Furthermore, p53 was strongly upregulated in lung cytosolic, nuclear and mitochondrial fractions from 12 hours after LPS instillation. Finally, LPS-treated animals showed an early increase in cleaved α-fodrin as well as translocation of apoptosis-inducing factor (AIF) to the nucleus, both indicating increased calpain activity which is an important mediator of caspase-independent cell death.

Conclusions: We demonstrate here for the first time that LPS-induced lung injury was characterized by early activation of the extrinsic apoptotic pathway and late activation of the intrinsic apoptotic pathway accompanied by increased p53 expression. In addition, LPS-challenge resulted in the activation of caspase-independent cell death pathways.

INTRODUCTION

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are severe illnesses characterized by increased vascular permeability, neutrophil accumulation and lung inflammation. Pro-inflammatory cytokines and subsequent generation of reactive oxygen intermediates may cause severe tissue damage and contribute to deterioration of lung function [1]. In addition, activation of cell death pathways during ALI has been suggested to contribute to loss of lung epithelial and endothelial cells and thereby to impaired functioning of the lung [2]. Several pathways leading to cell death have been described including caspase-dependent and caspase-independent pathways of cell death.

The caspase-dependent pathway of apoptosis is a process of controlled cell death and involves two main routes. The first route (also known as the intrinsic pathway of apoptosis) is initiated by environmental factors, such as changes in oxidation/reduction potential, and involves members of the Bcl-2 family. The Bcl-2 family comprises anti-apoptotic (Bcl-2, Bcl-xL) and pro-apoptotic (Bid, Bax, Bak and PUMA) members which mediate release of pro-apoptotic mitochondrial proteins, such as cytochrome *c*, into the cytoplasm [3]. In the cytoplasm, cytochrome *c* forms an apoptosome complex together with apoptotic protease-activating factor (APAF)-1 and cleaved caspase-9. This apoptosome cleaves caspase-3 leading to formation of the active form of this executioner of apoptosis and subsequently causing DNA fragmentation and cell death. The second pathway of apoptosis (extrinsic pathway) is initiated by ligand binding to members of the family of membrane bound tumor necrosis factor (TNF) receptors such as TNFR-1 and CD95 (Fas). Upon receptor activation by its ligand, caspase-8 is activated and this can directly lead to the activation of caspase-3. Importantly, there exists a cross-talk between the extrinsic and intrinsic apoptotic pathway through the cleavage of Bid by caspase-8, leading to formation of truncated Bid (tBid). tBid can bind to the mitochondria where it induces cytochrome *c* release from the mitochondria.

Several studies have suggested that the extrinsic pathway of apoptosis plays a pivotal role in the development of ALI. In this respect, alveolar epithelial injury in humans with ARDS is associated with local upregulation of the Fas/Fas ligand (FasL) system [4]. In addition, soluble FasL accumulates in bronchoalveolar lavage fluid (BALf) of patients with ARDS [5]. Furthermore, studies applying animal models of lung injury indicate that stimulation of Fas-dependent pathways causes alveolar epithelial apoptosis and lung injury [6,7]. Although the role of Fas-induced cell death has been intensively studied in ALI, the contribution of the intrinsic apoptotic pathway and other cell death mediators have been less well studied. For example, an important mediator of both the intrinsic and the extrinsic apoptotic pathway is p53, a tumor suppressor protein. P53 is a transcription factor for several Bcl-2 family genes, including Bax, PUMA and Bid, thereby promoting cytochrome *c* release from the mitochondria. In addition, p53 induces APAF-1 expression which is required for the formation of the apoptosome complex in combination with caspase-9 and cytochrome *c*. P53 also activates

the extrinsic apoptotic pathway through the induction of Fas and the activation of caspase-8 [8]. In conclusion, p53 seems to play a pivotal role during cell death.

Besides caspase-dependent cell death, caspase-independent cell death pathways mediated by calpains may play a role in ALI. Calpains regulate cleavage of the cytoskeletal protein α-fodrin in a calcium-dependent way resulting in enhanced destabilization of the cell and eventually cell death [9]. Furthermore, calpains are involved in the proteolytic cleavage of mitochondrial apoptosis-inducing factor (AIF) resulting in its release from the mitochondria and subsequent translocation to the nucleus thereby facilitating caspase-independent cell death [10].

The aim of present study was to investigate the relative contribution of several cell death signaling pathways in a widely used model for ALI, i.e. lipopolysaccharide (LPS)-induced lung injury.

MATERIAL AND METHODS

Animals

Experiments were performed in accordance with international guide lines and approved by the experimental animal committee of the University Medical Center Utrecht. Male Spraque Dawley rats (weighing 280 to 300 grams) were obtained from Harlan CPB (Horst, the Netherlands). Rats were fed a standard pellet diet (Hope Farms, Woerden, the Netherlands) and water *ad libitum*.

Study design

Rats were randomized into six groups (n = 6 to 8 per group). Rats were intratracheally instilled with either 1 mg/kg LPS derived from *Salmonella enteritidis* (Sigma Aldrich, St. Louis, MO) or 0.1 ml sterile saline as control (vehicle). At 4, 12 or 24 hours after LPS-challenge rats were sacrificed and lungs were removed, snap frozen and stored at -80°C for further analysis.

Western blotting

Lungs were pulverized using a liquid nitrogen-cooled mortar and pestle, and stored at -80°C. The fractions were processed by homogenization in buffer containing 70 mM sucrose, 210 mM mannitol, 5 mM HEPES, 1 mM EDTA and protease inhibitors (Sigma) using a Potter-homogenizer (Heidolph, Schwabach, Germany). Homogenates were incubated on ice for 30 minutes, followed by 10 minutes centrifugation at 800 *g* at 4°C, leading to a nuclear pellet (P1). Supernatants (S1) were collected and centrifuged at 10,000 *g* for 10 minutes at

4°C to obtain mitochondrion-free supernatant (cytosol protein fraction) and a mitochondrial pellet (P2).

Nuclear protein fraction was obtained by homogenizing nuclear pellets (P1) in buffer containing 10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA and 1 mM dithiothreitol (DTT) supplemented with protease inhibitors using a Potter-homogenizer. After 15 minutes incubation on ice, NP-40 was added (final concentration of 3.125%), the mixture was vortexed and centrifuged for 1 minute at 10,000 *g* at 4°C. The nuclear pellet was resuspended in ice-cold hypertonic nuclear extraction buffer containing 20 mM HEPES, 420 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and protease inhibitors, incubated on ice for 30 minutes and centrifuged for 5 minutes at 10,000 *g* at 4°C. The supernatant containing the nuclear proteins was collected and kept at -80°C until use.

Mitochondrial protein fraction was obtained by sonicating mitochondrial pellets (P2) in ice-cold buffer containing 50 mM Tris, 5 mM EDTA, 150 mM NaCl and protease inhibitors. Homogenates were incubated on ice for 30 minutes, centrifuged at 10,000 *g* for 15 minutes at 4°C and supernatants containing mitochondrial proteins were collected.

Protein concentration was determined using a protein assay (Bio-Rad Laboratories, Hercules, CA). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Hybond-C; Amersham Biosciences, Roosendaal, the Netherlands). Equal protein loading was verified by Ponceau-S staining. Cytosolic proteins were analyzed for α-fodrin (Biovision, Mountain View, CA), p53 (Cell Signaling, Danvers, MA), cleaved caspase-3 (Cell Signaling), cleaved caspase-9, caspase-8, truncated and total Bid and β-actin (all Santa Cruz Biotechnology, Santa Cruz, CA). Mitochondrial proteins were analyzed for p53, cytochrome *c* (BD Biosciences Pharmingen, San Jose, CA) and COX IV (Molecular Probes, Eugene, OR). Nuclear proteins were analyzed for p53, AIF (Sigma) and histon (Santa Cruz). Immunoreactivity was detected by enhanced chemiluminescense (ECL; Amersham). Band density was determined using a GS-700 Imaging Densitometer and analyzed with Quantity One Software (both Bio-Rad).

Statistical analysis

All data are presented as mean ± SEM. All parameters were analyzed by two-way ANOVA with Bonferroni post-hoc test. P-values less than 0.05 were considered statistically significant.

RESULTS

Intratracheal LPS induces caspase-dependent cell death in the lung

To determine whether intratracheal LPS leads to activation of the caspase-dependent pathway of apoptosis in the lung, we examined the change in cleaved caspase-3 in lung cytosolic fractions from 4 to 24 hours after LPS instillation. We observed increased levels of cleaved caspase-3 in cytosol from LPS-treated animals compared to saline-treated animals starting at 12 hours and being most pronounced at 24 hours after LPS instillation (figure 1).

Intratracheal LPS activates the intrinsic apoptotic pathway

To investigate whether LPS activated the intrinsic apoptotic pathway in the lung, we first analyzed cleaved caspase-9 (37 kDa) expression in lung cytosolic fractions. As shown in figure 2a, instillation of LPS induced a marked increase in the level of cleaved caspase-9 only at 12 hours after treatment indicating that caspase-9 was activated.

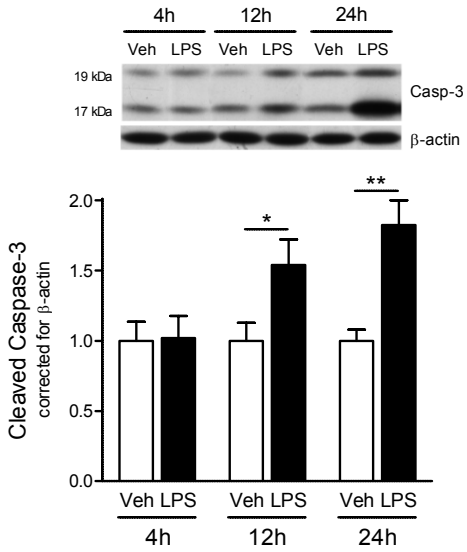

Figure 1: Effect of LPS on cleaved caspase-3 expression.
Cleaved caspase-3 expression levels were assessed in lung cytosolic fractions. Membranes were stripped and reprobed with antibody recognizing β-actin to control for equal loading. Inset: representative Western blot depicting immunodetectable cleaved caspase-3. Data are expressed as mean ± SEM, and depicted relative to control (vehicle). *p<0.05, **p<0.01.
Veh, LPS = intratracheally instilled with vehicle (sterile saline) or lipopolysaccharide; 4h, 12h, 24h = sacrificed at 4, 12 or 24 hours after intratracheal LPS; Casp = caspase.

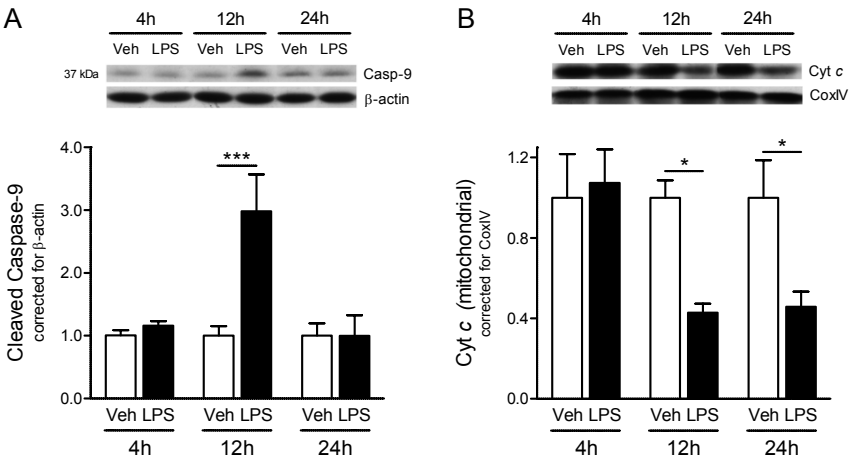

Figure 2: Effect of LPS on intrinsic apoptotic cell death.
A: Cleaved caspase-9 expression levels were assessed in lung cytosolic fractions. **B:** Cytochrome *c* expression levels were assessed in lung mitochondrial fractions. Membranes were stripped and reprobed with antibody recognizing β-actin (A) and CoxIV (B) to control for equal loading. Inset: representative Western blot depicting immunodetectable cleaved caspase-9 and cytochrome *c*. Data are expressed as mean ± SEM, and depicted relative to control (vehicle). *p<0.05, ***p<0.001.
Veh, LPS = intratracheally instilled with vehicle (sterile saline) or lipopolysaccharide; 4h, 12h, 24h = sacrificed at 4, 12 or 24 hours after intratracheal LPS; Casp = caspase; Cyt *c* = cytochrome *c*.

An important step in the activation of the intrinsic apoptotic pathway upstream from cleaved caspase-9 is the release of cytochrome *c* from the mitochondria. LPS-treated animals showed a significant reduction in mitochondrial cytochrome *c* expression compared to saline-treated animals at both 12 and 24 hours after LPS instillation (figure 2b).

Intratracheal LPS activates the extrinsic apoptotic pathway

To determine whether LPS also activated the extrinsic apoptotic pathway in the lung, we analyzed cleaved caspase-8 in lung cytosolic fractions. Western blot analysis revealed a time-dependent increase in cleavage of procaspase-8 to the 41 kDa and 18 kDa active subunits in LPS-treated lungs. The increase in cleaved caspase-8 was most pronounced at 4 hours after LPS instillation and declined over time (figure 3a). Lungs from saline-treated animals did not contain detectable levels of cleaved caspase-8.

One of the major downstream effects of active caspase-8 is the caspase-8–dependent cleavage of the pro-apoptotic Bcl-2 protein Bid into truncated Bid (tBid). In line with the increase in active caspase-8 at 4 hours after LPS instillation, we observed the most distinct enhancement in tBid at 4 hours after LPS which declined over time (figure 3b). Lungs from saline-treated animals did not contain detectable levels of tBid.

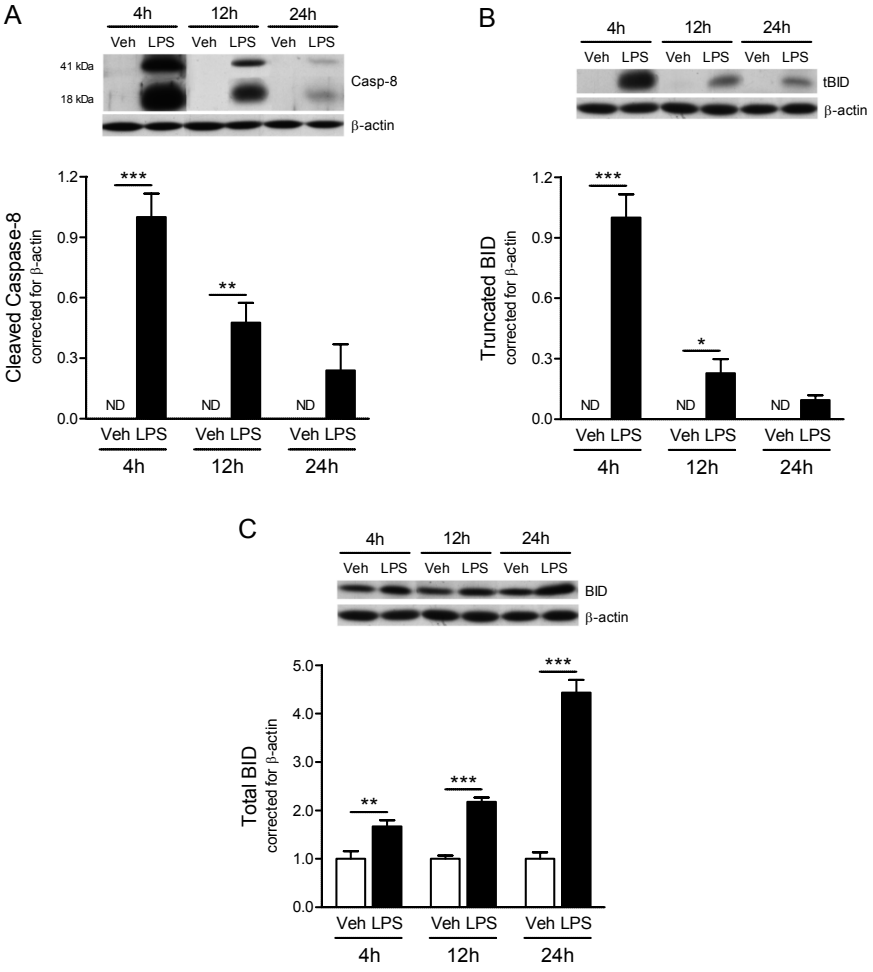

Figure 3: Effect of LPS on extrinsic apoptotic cell death.

A: Cleaved caspase-8 expression levels were assessed in lung cytosolic fractions. **B:** Truncated Bid (tBid) expression levels were assessed in lung cytosolic fractions. **C:** Total Bid expression levels were assessed in lung cytosolic fractions. Membranes were stripped and reprobed with antibody recognizing β-actin to control for equal loading. Inset: representative Western blot depicting immunodetectable cleaved caspase-8, truncated Bid and total Bid. Data are expressed as mean ± SEM, and depicted relative to control (vehicle). *p<0.05, **p<0.01, ***p<0.001.

Veh, LPS = intratracheally instilled with vehicle (sterile saline) or lipopolysaccharide; 4h, 12h, 24h = sacrificed at 4, 12 or 24 hours after intratracheal LPS; Casp = caspase.

Interestingly, levels of total Bid were also enhanced in LPS-treated animals compared to saline-treated animals both at 4 and 12 hours after LPS instillation and more pronounced at 24 hours after LPS instillation (figure 3c).

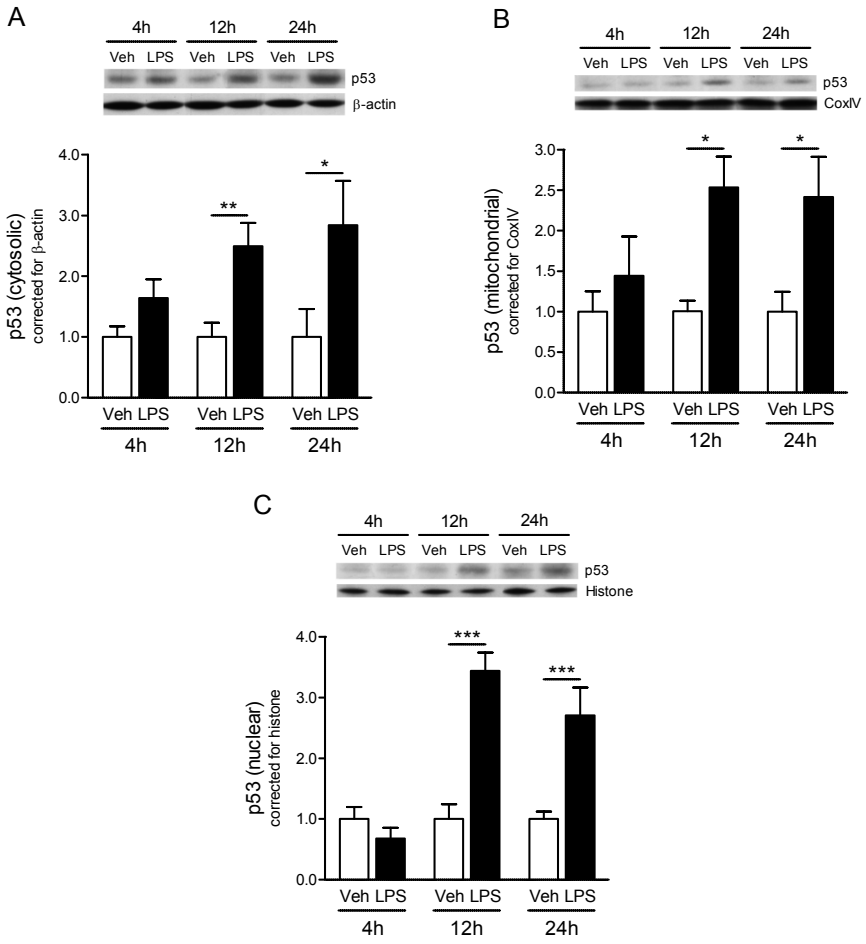

Figure 4: Effect of LPS on p53 expression.
A: p53 expression levels in lung cytosolic fractions. **B:** p53 expression levels in lung mitochondrial fractions. **C:** p53 expression levels in lung nuclear fractions. Membranes were stripped and reprobed with antibody recognizing β-actin (A), Cox IV (B) and histone (C) to control for equal loading. Inset: representative Western blot depicting immunodetectable p53. Data are expressed as mean ± SEM, and depicted relative to control (vehicle). *p<0.05, **p<0.01, ***p<0.001.
Veh, LPS = intratracheally instilled with vehicle (sterile saline) or lipopolysaccharide. 4h, 12h, 24h = sacrificed at 4, 12 or 24 hours after intratracheal LPS.

Intratracheal LPS increases p53 expression in the lung

An important mediator in the activation of the extrinsic and intrinsic apoptotic pathway is the tumor suppressor protein p53. Therefore, we analyzed p53 expression after LPS instillation in lung cytosolic, mitochondrial and nuclear fractions. In the cytosol, LPS-challenge resulted in a time-dependent increase of p53 compared to saline-treated animals starting at 4 hours

after LPS and being more pronounced at 12 and 24 hours (figure 4a). In the mitochondria, p53 was markedly upregulated in the LPS-treated animals at both 12 and 24 hours after LPS (figure 4b). Also in the nucleus we observed a late accumulation of p53 being significantly upregulated at 12 and 24 hours after LPS instillation compared to saline-treated animals (figure 4c).

Intratracheal LPS induces caspase-independent cell death pathways in the lung

To investigate the role of caspase-independent pathways after LPS instillation, we measured the level of cleaved α-fodrin in cytosolic fractions of lung homogenates as an indicator of calpain activity. As shown in figure 5a cleaved α-fodrin was significantly increased in the cytosol at 4 and 12 hours after LPS instillation.

A second important feature of calpain activity during cell death is the translocation of AIF from the mitochondria to the nucleus. Therefore, we determined AIF levels in nuclear fractions of the lung at 4, 12 and 24 hours after LPS instillation. At 4 hours after LPS no difference was observed in nuclear AIF between LPS-treated animals and saline-treated animals. At 12 and 24 hours, however, a marked increase in nuclear AIF was observed in the LPS-treated animals compared to saline-treated animals (figure 5b).

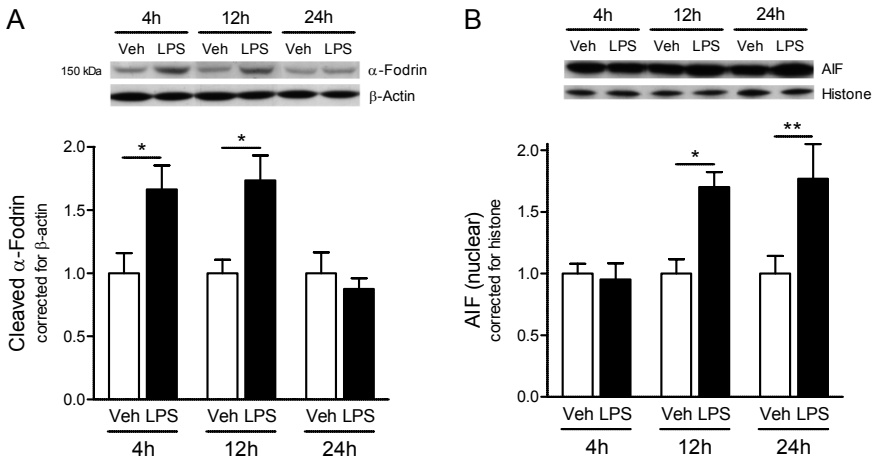

Figure 5: Effect of LPS on caspase-independent cell death.
A: Cleaved α-fodrin expression levels were assessed in lung cytosolic fractions. **B:** Apoptosis inducing factor (AIF) expression levels were assessed in lung nuclear fractions. Membranes were stripped and reprobed with antibody recognizing β-actin (A) and histone (B) to control for equal loading. Inset: representative Western blot depicting immunodetectable cleaved α-fodrin and AIF. Data are expressed as mean ± SEM, and depicted relative to control (vehicle). *p<0.05, **p<0.01.
Veh, LPS = intratracheally instilled with vehicle (sterile saline) or lipopolysaccharide; 4h, 12h, 24h = sacrificed at 4, 12 or 24 hours after intratracheal LPS.

DISCUSSION

In this study we demonstrated that intratracheal LPS activated not only the extrinsic apoptotic pathways but also the intrinsic apoptotic pathway in the lung which was associated with increased nuclear, mitochondrial and cytosolic p53. In addition, we showed that LPS instillation activated caspase-independent cell death pathways as we observed an increase in AIF in the nucleus and cleaved α-fodrin in the cytosol. These cell death mechanisms may play a pivotal role in lung diseases such as ALI.

ALI is an inflammatory disorder of the lung caused by pulmonary events such as pneumonia and aspiration or by extra-pulmonary events such as trauma and sepsis [11]. ALI is characterized by impaired gas exchange, increased influx of neutrophils and increased pulmonary vascular permeability. One of the underlying mechanisms involved in ALI may be enhanced endothelial/epithelial cell death [12]. We used an *in vivo* rat model of ALI by instilling LPS intratracheally. LPS instillation in the lung mimics many of the features of ALI including the release of pro-inflammatory cytokines, organ failure and cell death [13].

Several studies have shown that during ALI the Fas/FasL system plays a pivotal role in the apoptosis signaling system suggesting that the extrinsic pathway is activated in lung cells [4,7,14]. We showed here that active caspase-8, an important caspase in the Fas/FasL system, is upregulated in the lung at 4 hours after LPS instillation. Also at later time points, 12 and 24 hours after LPS, active caspase-8 is still present although to a lesser extent. Therefore, we conclude that the apoptotic extrinsic pathway is rapidly activated and declines over time. It has been suggested that the activation of the extrinsic pathway by LPS is mediated by the upregulation of TNF-α and FasL which are known to activate death receptors on the surface of cells [15,16]. However, a direct effect of LPS on the activation of the extrinsic pathway via Toll-like receptor (TLR)4 and CD14 cannot be ruled out [17]. Future research using $TLR4^{-/-}$ animals can further unravel the mode of action of LPS in this model.

The activation of the extrinsic pathway via active caspase-8 is known to induce cell death via the executioner of apoptosis, caspase-3. We demonstrate here that pulmonary active caspase-3 was upregulated from 12 hours after LPS instillation and even more pronounced at 24 hours after LPS. Interestingly, we are the first to show activation of the intrinsic cell death in LPS-induced lung injury as represented by a significant increase in activated caspase-9 at 12 hours after LPS instillation, which is the primary caspase activated by the intrinsic pathway. Prior to caspase-9 activation, the mitochondrial outer membrane is permeabilized resulting in the release of cytochrome *c* from the mitochondrial intermembrane space. As shown in our study, intratracheal LPS induced release of cytochrome *c* from the mitochondria from 12 hours after LPS. Several pathways have been described that induce mitochondrial outer membrane permeabilization leading to release of cytochrome *c*, activation of caspase-9 and subsequent activation of caspase-3. First of all, the pro-apoptotic protein Bid may result in permeabilization of the mitochondrial membrane. Full-length Bid is inactive, but after

caspase-8–mediated cleavage, truncated Bid induces cytochrome *c* release from the mitochondria. We report here that truncated Bid is enhanced upon LPS instillation already starting at 4 hours after LPS concomitantly with the activation of caspase-8. In line with this finding Wang et al. showed that intraperitoneal LPS induced less injury and apoptosis in $Bid^{-/-}$ mice compared to wild-type mice [18]. The second explanation for mitochondrial permeabilization may be a role for the tumor suppressor protein p53. Many studies have shown that p53 possesses both transcription-dependent and transcription-independent activities. P53 activates the intrinsic pathway by acting directly at mitochondria thereby promoting mitochondrial outer membrane permeabilization [8]. We observed an increased expression of p53 in the mitochondria at 12 and 24 hours after LPS instillation. Therefore, p53 translocation to mitochondria leading to mitochondrial permeabilization and leakage may well be the mechanism in LPS-induced pulmonary cell death. In addition, p53 can activate the intrinsic pathway by upregulating the expression of pro-apoptotic proteins such as Bax and Bid. In line with this, we observed increased levels of p53 in the nucleus at 12 and 24 hours after LPS. In the nucleus p53 acts as a transcription factor and may therefore be responsible for the increased expression of full-length Bid as observed in our experiments. We also observed LPS-induced upregulation of p53 in the cytoplasm of pulmonary cells already starting at 4 hours after LPS. Ding et al. have shown that cytoplasmic p53 activates caspase-8, thereby promoting the extrinsic pathway [19]. Therefore, we propose that p53 may be an important mediator in LPS-induced pulmonary cell death promoting both the intrinsic and extrinsic apoptotic pathway. Interestingly, Liu et al. described that neutrophils and macrophages from $p53^{-/-}$ mice had elevated inflammatory responses to LPS compared to wild-type mice [20]. Therefore, it could be possible that treatment with a p53 inhibitor may decrease apoptotic cell death in lungs, but increase inflammation in macrophages and neutrophils. Future research needs to be performed to further delineate these dual effects of p53.

We also investigated caspase-independent cell death pathways induced by calpains. Calpains are calcium-dependent cysteine proteases and involved in many cellular events including cell death. Increased intracellular levels of calcium play an important role in the influx of neutrophils and permeabilization of endothelial cells, thereby contributing to ALI [21,22]. The calcium influx activates several proteins such as calpains resulting in increased cell death. We show here for the first time that LPS induced an early increase in cleaved α-fodrin. Cleavage of α-fodrin is dependent on calpain activity and cleaved α-fodrin is known to contribute to caspase-independent cell death. Another important consequence of the activation of calpains is the translocation of AIF from the mitochondria to the nucleus resulting in caspase-independent cell death [10]. In line with this notion, we showed for the first time increased nuclear AIF after LPS instillation in the lung. On the basis of our results we would like to propose that calpains may contribute to cell death associated with LPS-induced lung injury.

There are several limitations to the current data. First of all, the cellular source of the cell death signaling pathways is unknown. It may well be that the observed changes result from alterations in the cellular make-up of the lung due to migration of various cells into the lung. On the other hand, LPS could also induce distinct changes in the intrinsic signaling pathways of specific cell types. Therefore, in future experiments we will focus on the LPS-induced cell death pathways in different lung cell types.

Another limitation of our study is that specific knock-out animals, like $Bid^{-/-}$ or $p53^{-/-}$ mice, are necessary to further assess the role of these products in the various cell death pathways in our model. However, in our study we used rats so future studies will be performed in mice.

In conclusion, we described in this paper that LPS-induced lung injury in rats is associated with early activation of the extrinsic apoptotic pathway and late activation of the intrinsic apoptotic pathway. In addition, p53 was strongly upregulated in cytosol, nucleus and mitochondria from pulmonary cells suggesting that p53 may contribute to LPS-induced lung injury. Finally, LPS instillation resulted in the activation of caspase-independent cell death pathways. Further experiments are needed to delineate the functional role of these apoptotic processes in lung diseases such as ALI.

CONCLUSIONS

We show here for the first time that LPS-induced lung injury was characterized by early activation of the extrinsic apoptotic pathway and late activation of the intrinsic apoptotic pathway accompanied by increased p53 expression. In addition, LPS-challenge resulted in the activation of caspase-independent cell death pathways. Therefore, inhibiting caspase-dependent cell death via p53 and/or inhibiting caspase-independent cell death via calpain may be a good approach to treat inflammatory lung diseases such as ALI.

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CHAPTER

7

General discussion & Summary

GENERAL DISCUSSION

Mechanical ventilation has the potential to induce or aggravate damage to lung tissue, so-called ventilator-induced lung injury (VILI) [1,2]. Recently, it has been recognized that VILI may not only occur after "injurious" ventilation strategies with high tidal volumes but also after "protective" ventilation strategies with low tidal volumes which are meant to preserve alveolar integrity. Apart from improving current ventilation strategies, additional therapies to prevent detrimental ventilator-induced effects on the lung are urgently needed. In this thesis, we first describe possible mechanisms that may underlie the pathogenesis of VILI. The main focus of this thesis is to evaluate if and how different therapeutic interventions attenuate various aspects of VILI like inflammation, alveolar-capillary permeability and impaired gas exchange.

In this last chapter, we will discuss our most intriguing data and speculate about the applicability of potential therapeutic intervention strategies to protect critically ill patients against the detrimental effects of mechanical ventilation.

Mechanical ventilation of healthy mice: lung and distal organ inflammation

Leukocyte-endothelial interactions have been recognized to be important in the pathogenesis of serious inflammatory diseases related to VILI, such as acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) [3,4]. Moreover, these interactions have been shown to be play a crucial role in systemic events like sepsis and multiple organ failure (MOF) [5]. Therefore, our first question was whether alveolar stretch caused by mechanical ventilation results in endothelial activation and inflammation in healthy mice, not only in the lung but also in organs distal to the lung (**chapter 2**). We observed that 4 hours of mechanical ventilation with high pressures, i.e. high level of alveolar stretch, induced *de novo* synthesis of various adhesion molecules not only in the lung but also in the liver and kidney. [6]. Moreover, increased cytokine and chemokine mRNA levels were found in the lung and distal organs of ventilated mice which was accompanied by enhanced granulocyte infiltration. Our data imply that ventilator-induced endothelial activation in the lung, liver and kidney facilitates migration and adhesiveness of activated immune cells to inflamed tissue, which in turn may lead to tissue injury in these organs.

Since we observed an increased pro-inflammatory response after mechanical ventilation in both the lung and distal organs, it is tempting to speculate that mechanical ventilation may play a significant role in the development of MOF. Supporting this hypothesis, earlier experimental research provided evidence that mechanical ventilation evokes detrimental effects in distal organs [7,8]. Imai et al. showed that mechanical ventilation with high tidal volumes induces epithelial cell apoptosis in the kidney and small intestine [7]. Moreover, they demonstrated that mechanical ventilation leads to elevated levels of serum markers such as creatinine indicative for renal failure. Ventilator-induced cardiovascular and hepatic injury

have been described as well [8]. It should be noted that these previous studies used animals with *pre-existing* lung injury whereas our study used *healthy* animals, suggesting that already existing inflammation is not a prerequisite for unveiling the negative effects of mechanical ventilation on distal organs.

It remains to be determined which underlying mechanisms may initiate the onset of inflammatory responses in distal organs during mechanical ventilation. Haitsma et al. and Tutor et al. showed that ventilator-induced permeability of the alveolar-capillary barrier leads to release of inflammatory mediators into the systemic circulation [9,10]. We demonstrate in this thesis, however, that ventilator-induced changes on the level of adhesion molecule, cytokine and chemokine expression may occur simultaneously in the lung, liver and kidney [6]. In view of this spatiotemporal pattern of inflammatory mediator expression, we suggest that release of cytokines and chemokines from the lung into the circulation is probably not the only cause of the enhanced pro-inflammatory environment in distal organs during mechanical ventilation. Yet, we cannot exclude that inflammatory mediators released into the circulation may contribute to *de novo* synthesis of adhesion molecules, cytokines and chemokines in distal organs, since cytokines such as IL-1β and TNF-α have been shown to induce and stimulate production of a host of inflammatory mediators [11].

Previously, it has been described that the physical stress of mechanical ventilation activates the sympathetic nervous system and leads to an increase in catecholamines [12]. Several investigators have proposed that stimulation of sympathetic nerve terminals may evoke a pro-inflammatory response in peripheral organs [13,14]. In this respect, Elenkov et al. reported that locally released catecholamines may activate transcription factors, like NF-κB, in macrophages thereby promoting IL-1β, TNF-α and IL-8 production [13,15,16]. Based on this notion, we would like to propose that ventilator-induced activation of sympathetic nerve terminals in distal organs may also contribute to the pro-inflammatory state of these organs. This pro-inflammatory state of distal organs may prime for the development of MOF in the critically ill patient [7,17,18]

Besides an enhanced pro-inflammatory systemic environment, it has been proposed that suppression of the peripheral immune system may be crucially involved in the pathogenesis of MOF as well [19,20]. Initially, suppression of the peripheral immune system may provide a compensatory mechanism to restore homeostasis [21]. However, the compensatory reaction may become maladaptive when existing for prolonged periods of time consequently leading to excessive suppression of the peripheral immune system and augmented susceptibility to infections. In this respect, Angele and Faist reported that many ventilated critically ill patients suffer from unexplained immune suppression and an associated increased risk for infections and MOF [22]. Previously, we demonstrated both clinically and experimentally that mechanical ventilation suppresses leukocyte function outside the lung, i.e. decreased natural killer cell activity, cytokine production and mitogen-induced splenocyte proliferation [23,24]. Supporting these previous findings, preliminary data on our murine model of VILI demonstrated

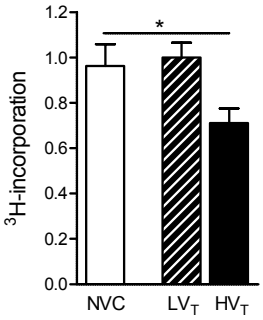

Figure 1: Ventilator-induced peripheral immune suppression.
In vitro mitogen-induced splenocyte proliferation was assessed as a measure of peripheral immune suppression. Splenocytes were stimulated *in vitro* with αCD3 (1μg/ml). After 48 hours ^{3}H-thymidine was added and 16 hours later incorporation of ^{3}H-thymidine was determined. Data are expressed as mean ± SEM, and depicted relative to LV_T. NVC n=10, LV_T n=16, HV_T n=13. * $p<0.05$; ANOVA with least significant difference (LSD) post-hoc test.
NVC = non-ventilated controls; LV_T, HV_T = mechanically ventilated with low or high tidal volumes.

that mechanical ventilation with high tidal volumes inhibited mitogen-induced splenocyte proliferation compared to non-ventilated controls (NVC) as well (figure 1).

Our data indicate that mechanical ventilation does not only induce a pro-inflammatory environment in organs distal to the lung but also impairs the functioning of the peripheral immune system (peripheral immune suppression). The suppressed immune functioning during mechanical ventilation may increase the patient's susceptibility to infections and consequently contribute to MOF.

Mechanical ventilation of healthy mice: lung injury

Mechanical ventilation is known to destabilize the alveolar-capillary barrier thereby leading to increased alveolar-capillary permeability, pulmonary edema formation and impaired gas exchange [25-27]. Loss of integrity of epithelial and endothelial monolayers is thought to play an important role in ventilator-induced disruption of alveolar-capillary barriers [28]. One of the crucial systems regulating vascular cell integrity is the angiopoietin (Ang)-Tie2 system [29]. Previous clinical and experimental studies demonstrated the importance of the Ang-Tie2 system in the pathogenesis of lung diseases related to VILI, like ALI/ARDS [30]. Karmpaliotis et al. have proposed that changes in the balance between vascular endothelial growth factor (VEGF) (pro-leakage) and Ang-1 (anti-leakage) might contribute to vascular leakage in a murine model of lipopolysaccharide (LPS)-induced ALI [31]. Based on these notions, we examined whether 5 hours of mechanical ventilation with low or high tidal volumes (LV_T or HV_T-ventilation) would influence the Ang-Tie2 system and VEGF expression in lungs of

healthy adult mice (**chapter 5**). Particularly in lungs of HV_T-ventilated mice, marked changes were observed in the Ang-Tie2 system. We demonstrated that Ang-1, Ang-2 and Tie2 mRNA expression was significantly reduced in HV_T-ventilated mice in comparison with NVC. At this time point, Ang-1 and Ang-2 protein expression also tended to decrease. Moreover, we found that HV_T-ventilation caused increased expression of VEGF which is in agreement with a prior report [32]. Our findings strongly suggest that changes in the Ang-Tie2 system, together with increased VEGF expression, are involved in the development of lung injury during mechanical ventilation.

Besides loss of cell integrity, pulmonary cell death might be involved in the ventilator-induced disruption of alveolar-capillary barriers [28]. In an *in vitro* model of alveolar stretch, Hammerschmidt et al. demonstrated that mechanical stretch activated apoptotic and necrotic cell death [33]. Therefore, we investigated whether cell death pathways were activated in our murine model of VILI. As measures of caspase-dependent cell death, we determined protein expression of cleaved caspase-8, -9 and -3. Our data show unequivocally that basal cleavage of caspase-8 was not affected by either LV_T or HV_T-ventilation (figure 2a). Furthermore, cleaved caspase-9 and -3 were below detection level in all experimental groups implying that caspase-dependent apoptotic pathways were not activated by LV_T or HV_T-ventilation.

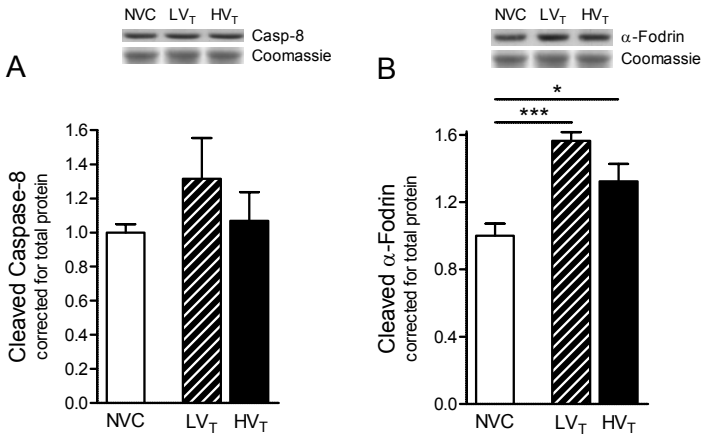

Figure 2: Effect of mechanical ventilation on caspase-dependent and caspase-independent pathways of cell death.

A: In total lung homogenates, protein expression of cleaved caspase-8 was determined by Western blotting as a measure for caspase-dependent cell death. Cleaved caspase-9 and -3 were below detection level in all experimental groups. **B:** In addition, protein expression of cleaved α-fodrin was determined as a measure of caspase-independent cell death. Levels were normalized for total protein levels (Coomassie staining). Inset: representative Western blot depicting immunodetectable cleaved caspase-8 and α-fodrin. Data are expressed as mean ± SEM, and depicted relative to NVC. A-B: NVC n=6, LV_T n=7, HV_T n=8. * $p<0.05$, *** $p<0.001$; ANOVA with least significant difference (LSD) post-hoc test.

NVC = non-ventilated controls; LV_T, HV_T = mechanically ventilated with low or high tidal volumes.

Elevated intracellular calcium levels may lead to activation of several proteins, including calpains, which in turn cleave the cytoskeletal protein α-fodrin and cause cell destabilization and (caspase-independent) necrosis [34]. We observed that both LV_T and HV_T-ventilation increased levels of cleaved α-fodrin in comparison to NVC (figure 2b). On the basis of these findings, it is tempting to speculate that 5 hours of mechanical ventilation induces cell death primarily via caspase-independent necrotic pathways. In contrast to our data, Wolthuis et al. demonstrated in a similar model of VILI that the number of caspase-3 positive cells on lung sections was higher in HV_T-ventilated mice than in NVC although this increase did not reach statistical significance (p=0.055) [35]. Taken together, we propose that apoptosis is not the major executive mechanism of cell death in our model of VILI though we cannot exclude that caspase-dependent pathways of cell death are modestly activated.

On the basis of these data, we suggest that changes in the Ang-Tie2 system and caspase-independent cell death (necrosis) may be important in the impairment of alveolar-capillary barrier function during mechanical ventilation.

Liposome-encapsulated dexamethasone: does it attenuate ventilator-induced lung inflammation?

It has been recognized that granulocytes and inflammatory mediators play a crucial role in the pathogenesis of VILI [36-38]. As a consequence, anti-inflammatory agents like synthetic glucocorticoids have been used in the intensive care unit to attenuate or prevent detrimental effects induced by mechanical ventilation. However, the efficacy of synthetic glucocorticoids to treat ALI/ARDS in critically ill patients is still under debate [39-41].

Although glucocorticoids have the potential to inhibit ventilator-induced lung inflammation [42,43], systemic administration may cause severe side effects like increased blood glucose levels [44,45]. Drug delivery systems aimed at preventing these unwanted systemic side effects could therefore be of therapeutic importance. Previously, Ásgeirsdóttir et al. have clearly shown that delivery of liposome-encapsulated dexamethasone downregulated inflammatory gene expression without increasing blood glucose levels, in contrast to administration of free dexamethasone [46]. In **chapter 3**, we studied if liposomes containing dexamethasone (Dex-liposomes) inhibited ventilator-induced lung inflammation. Especially in LV_T-ventilated mice, we observed that Dex-liposomes diminished important inflammatory parameters of VILI such as IL-1β, IL-6 and KC mRNA expression in the lung. Nonetheless, this liposomal formulation was not as effective as free dexamethasone in preventing granulocyte infiltration into pulmonary tissue. Since granulocytes are known to be crucially involved in the pathogenesis of VILI [47], we proposed that phagocytosis of liposomes by *activated* granulocytes may be beneficial and should therefore be enhanced. We hypothesized that IgG-modified Dex-liposomes (IgG-Dex-liposomes) may be more efficient in reducing ventilator-induced lung

inflammation than Dex-liposomes due to interaction with Fcγ-receptors (FcγRs) on activated granulocytes and macrophages [48]. Indeed, IgG-Dex-liposomes were pharmacologically more effective than Dex-liposomes particularly in preventing granulocyte infiltration induced by LV_T or HV_T-ventilation. In addition, IgG-Dex-liposomes inhibited ventilator-induced granulocyte infiltration and cytokine/chemokine expression as effective as free dexamethasone. Our data imply that conjugation of IgG to Dex-liposomes markedly improves their efficacy in protecting against ventilator-induced lung inflammation. Moreover, the use of liposomes may favour the local release of dexamethasone thereby preventing unwanted systemic side effects [46].

Inhibiting lung inflammation by dexamethasone: does it preclude ventilator-induced lung injury?

At present, it is thought that a ventilator-induced inflammatory response in the lung may precede pulmonary injury [36-38]. Activated granulocytes will initiate oxidative stress and protease activity in the alveoli, thereby causing severe disruption of pulmonary epithelial-endothelial barriers and leading to impaired oxygenation [3,49,50]. As described in **chapters 3 and 4**, we observed that both LV_T and HV_T-ventilation caused increased cytokine, chemokine and adhesion molecule expression accompanied by significant granulocyte influx compared to NVC. Bronchoalveolar lavage fluid (BALf) neutrophil numbers and inflammatory mediator levels were even higher when comparing lungs of HV_T-ventilated mice to lungs of LV_T-ventilated mice (**chapter 4**). Since deteriorated PaO_2/FiO_2 ratios were only observed after HV_T-ventilation, it may well be that ventilator-induced lung inflammation ultimately leads to impaired gas exchange. Our next question was, therefore, whether downregulation of lung inflammation by dexamethasone attenuates vascular leakage and impaired gas exchange induced by mechanical ventilation (**chapter 4**). In line with previous studies [42,43], we showed that treatment with dexamethasone completely inhibited the inflammatory response caused by 5 hours of mechanical ventilation. Moreover, dexamethasone administration decreased VEGF expression in lungs of LV_T and HV_T-ventilated mice. Despite significant reduction of lung inflammation and VEGF expression, dexamethasone did not diminish the more crude parameters of VILI such as the increase in BALf protein level, the increase in pulmonary wet-to-dry ratio and the reduction in PaO_2/FiO_2 ratio.

Our findings are in apparent contrast with previous studies showing protective effects of glucocorticoid therapy on lung injury [8,43]. In a rat model of VILI, Ohta et al. demonstrated that administration of glucocorticoids caused a marked leftward shifting of the pressure-volume (P-V) curve of rats ventilated for 40 minutes [43]. Yet, the deterioration of the P-V curve was still evident regardless of significant reduction in granulocyte influx. Ohta et al. explained their data by the effects of mechanical stretch on lung tissue, such as stress failure of pulmonary capillaries, which may contribute to lung injury to a great extent [51]. Since

mice were exposed to 5 hours of mechanical ventilation in our model of VILI, it is tempting to speculate that the progressive lung injury induced by prolonged mechanical stretch may not be influenced by the anti-inflammatory action of dexamethasone anymore.

In view of these data, we would like to suggest that dexamethasone treatment does not prevent the aspects of VILI driven by mechanosensitive alterations in barrier properties [52] but will only regulate the pulmonary inflammation. Therefore, reducing the effect of mechanical stretch on lung injury solely by anti-inflammatory intervention might be difficult.

Treatment with angiopoietin (Ang)-1: does it prevent ventilator-induced lung injury?

Loss of epithelial and endothelial cell integrity has been recognized to be a prominent feature of VILI [28]. As mentioned before, our data suggest that changes in the Ang-Tie2 system, together with increased VEGF expression, are involved in the development of lung injury during mechanical ventilation.

The Ang-Tie2 system has been proposed as a possible therapeutic target in serious pulmonary diseases related to VILI, like ALI/ARDS [30,53]. Therefore, we examined whether treatment with Ang-1 (a Tie2 receptor agonist) protects HV_T-ventilated mice against vascular leakage and impaired gas exchange (**chapter 5**). We observed that Ang-1 administration attenuated granulocyte infiltration and inflammatory mediator expression induced by 5 hours of HV_T-ventilation. In this respect, it is of interest that Ang-1 also exerts anti-inflammatory effects in experimental models of endotoxin-induced ALI [54-56]. Mei et al. showed that treatment with Ang-1 inhibited granulocyte infiltration by reducing chemotactic, adhesive and pro-inflammatory mediators in lungs of endotoxin-challenged, non-ventilated mice [57].

Previously, Nin et al. demonstrated that increased alveolar-capillary permeability and pulmonary edema formation due to mechanical ventilation are associated with elevated VEGF concentration in BALf [32]. Similarly, we observed that HV_T-ventilation induced VEGF expression in lung tissue. Thurston et al. described that vascular leakage induced by VEGF may be counteracted by Ang-1 [58,59]. Indeed, we showed that Ang-1 treatment completely abolished the increase in VEGF protein induced by HV_T-ventilation. Despite significant attenuation of lung inflammation and VEGF expression, treatment with Ang-1 did not prevent vascular leakage and impaired gas exchange. In apparent contrast, protective effects of Ang-1 on vascular leakage have been shown in endotoxin-challenged animals [54-57]. An explanation for this discrepancy might be that in HV_T-ventilation, the enhanced inflammation is not the primary inducer of lung injury as is the case in the induction of lung injury by LPS. In HV_T-ventilation, the primary functional deficit may be more geared at early cellular death due to mechanical (over)stretch, which will probably not be counteracted by Ang-1 treatment.

*Taken together, we propose that prevention of lung inflammation does not preclude a loss of pulmonary function in our experimental model of VILI, as has been shown in experimental models of endotoxin-induced ALI. Most likely, the injury caused by mechanical ventilation is different from the injury caused by endotoxin-challenge. In this respect, it is of interest that endotoxin-challenge provokes lung injury via caspase-dependent apoptosis and caspase-independent necrosis (**chapter 6**), whereas lung injury induced by mechanical ventilation is for the greater part caspase-independent (figure 2) and probably executed by pathways involving a destabilization of the cytoskeleton via proteolytic activity of calpains.*

IL-6: possible role in pathogenesis of ventilator-induced lung injury

Release of the pro-inflammatory cytokine IL-6 during mechanical ventilation has been described both in clinical and experimental studies [60,61]. In agreement with these previous reports, we observed significantly elevated levels of IL-6 after mechanical ventilation especially in lungs of HV_T-ventilated mice (**chapters 3, 4 and 5**). Interestingly, we observed that IgG-Dex-liposomes and Dex-liposomes did not influence IL-6 expression induced by HV_T-ventilation whereas both completely prevented the increase in IL-1β (**chapter 3**). Our data may indicate that the target cells of these liposomal formulations, primarily activated macrophages and granulocytes, may not be the only source of IL-6 production. Indeed, it has been described that IL-6 may be derived from a wide variety of pulmonary cell types [62]. *In vitro* studies demonstrated that alveolar epithelial and capillary endothelial cells are also activated by mechanical stretch, in a stretch-amplitude dependent matter, thereby releasing inflammatory mediators into the surrounding lung tissue [63,64]. Iwaki et al. observed that high cyclic stretch of microvascular endothelial cells resulted in increased production of IL-6 [63]. Moreover, Vlahakis et al. found that mechanical stretch induced cytokine release by alveolar epithelial cells as well [64]. Since IL-6 mRNA expression remained high despite treatment with IgG-Dex-liposomes or Dex-liposomes, it is tempting to speculate that alveolar epithelial and capillary endothelial cells will not internalize these liposomes and will therefore be responsible for the lack of IL-6 downregulation. The fact that both liposomal formulations effectively inhibited IL-1β and KC, which are primarily produced by alveolar macrophages [62], supports this explanation.

In inflammation, endothelial cells are known to play an important role in recruitment of immune cells to affected tissue, for instance via expression of adhesion molecules like E-selectin [65]. In this respect, we have shown that mechanical ventilation induces upregulation of E-selectin in lung tissue which was accompanied by increased granulocyte influx (**chapter 2**). To specifically target dexamethasone into activated endothelial cells, we also conjugated anti-E-selectin to Dex-liposomes (immunoliposomes). However, an improvement with respect to inhibition of lung inflammation was not observed when comparing immunolipsomes to IgG-Dex-liposomes (data not shown). The only difference we found was

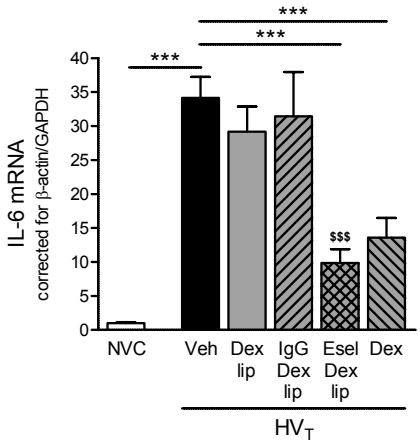

Figure 3: Effect of Dex-liposomes, IgG-Dex-liposomes, anti-E-selectin Dex-liposomes and free dexamethasone on IL-6 mRNA expression induced by HV_T-ventilation.
In total lung homogenates, mRNA expression of interleukin (IL)-6 was determined by real-time RT-PCR. Levels were normalized for expression of internal controls, i.e. the average value of β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPdH). Data are expressed as mean ± SEM, and depicted relative to NVC. NVC n=15, HV_T Veh n=18, HV_T Dex lip n=13, HV_T IgG-Dex lip n=10, HV_T Esel-Dex lip n=12, HV_T Dex n=7. *** $p<0.001$ versus NVC or HV_T Veh, $^{\$\$\$}$ $p<0.001$ versus HV_T Dex lip or HV_T IgG-Dex lip; ANOVA with least significant difference (LSD) post-hoc test.
NVC = non-ventilated controls; HV_T = mechanically ventilated with high tidal volumes; Veh, Dex lip, IgG-Dex lip, Esel-Dex lip, Dex = intravenously treated with either vehicle (sterile saline), liposomes containing dexamethasone, IgG-modified liposomes containing dexamethasone, anti-E-selectin modified liposomes containing dexamethasone (immunoliposomes), or free dexamethasone.

that immunoliposomes inhibited IL-6 expression induced by HV_T-ventilation in contrast to IgG-Dex-liposomes (figure 3). Therefore, it may well be that endothelial cells are the main source of IL-6 production during mechanical ventilation. It still remains to be established why Ang-1 treatment did not result in decreased IL-6 expression even though Ang-1 primarily acts on endothelial cells (**chapter 5**) [66,67].

It appears that the pro-inflammatory cytokine IL-6 is less responsive to treatment with dexamethasone or Ang-1 than other inflammatory mediators (like IL-1β). Therefore, we would like to suggest that the role of IL-6 may be crucially involved in the development of lung injury during HV_T-ventilation. For future therapeutic interventions, it would be of importance to delineate the precise source of IL-6 in the pathogenesis of VILI.

Future therapeutic interventions in critically ill patients with VILI

The recognition that conventional mechanical ventilation may provoke detrimental effects has led to the introduction of "lung-protective" ventilation strategies [68]. So far, mechanical ventilation with reduced tidal volumes is the only therapeutic approach effectively attenuating pulmonary injury and subsequent mortality in critically ill patients with ALI/ARDS [69]. In this regard, the ARDS Network revealed a 22% relative risk reduction in mortality rate when ventilating ALI/ARDS patients with "lung-protective" strategies [69]. Recent research has provided striking evidence that even "lung-protective" mechanical ventilation may induce the development of important parameters of VILI [70-72]. Our data from **chapters 3 and 4** confirmed these earlier findings. In our murine model of VILI, we observed that not only conventional ventilation strategies with high tidal volumes (HV_T-ventilation) but also clinically relevant ventilation strategies with low tidal volumes (LV_T-ventilation) increased inflammation and vascular leakage in the lung. Thus, the use of additional therapeutic interventions might contribute to the survival of critically ill patients.

In **chapters 4 and 5** we described that inhibition of ventilator-induced lung inflammation does not prevent the development of more crude parameters of VILI, like the increase in BALf protein level, the increase in pulmonary wet-to-dry ratio and the reduction in PaO_2/FiO_2 ratio. Based on these results, we would like to propose that anti-inflammatory agents should not be used to combat the mechanosensitive aspects of ventilator-induced lung injury. However, anti-inflammatory therapy may well be considered when inflammation is the primary inducer of lung injury, like in non-ventilated patients diagnosed with ALI/ARDS.

The findings in this thesis suggest that future therapeutic interventions in the ventilated, critically ill patient should aim at attacking the ventilator-induced impairment of alveolar-capillary barrier function. Stem cells have been demonstrated to restore function of damaged tissue in experimental models of lung disease [57,73,74]. Moreover, genetic engineering of stem cells has been shown to improve the protective effects of stem cells even more [57]. Mei et al. evaluated the integrity of the alveolar-capillary membrane barrier by measuring BALf total protein, albumin and IgM concentration in a murine model of LPS-induced ALI. They showed that treatment with stem cells alone already partially reduced these indicators of lung injury in LPS-exposed mice. Stem cells overexpressing Ang-1, however, restored total protein, albumin and IgM to levels not different from naïve control animals. It should be noted, though, that mechanical ventilation provokes a different spectrum of injury compared to LPS-challenge as demonstrated by the difference in efficacy of Ang-1 treatment in our model of VILI (**chapter 5**).

Pulmonary cell death is thought to be involved in the ventilator-induced disruption of alveolar-capillary barriers and thus in the impairment of alveolar-capillary barrier function [28]. In **chapter 6** we evaluated whether different pathways of cell death may be activated in LPS-induced ALI, an experimental model related to VILI. LPS instillation in the lung is

known to mimic many of the features of ALI - including release of pro-inflammatory cytokines, organ failure and cell death - and is therefore considered as a suitable model for acute respiratory failure [75]. We demonstrated that intratracheal instillation of LPS caused elevated levels of cleaved caspase-8, -9 and -3 accompanied by increased expression of p53, an important mediator of both the intrinsic and extrinsic apoptotic pathway. Moreover, we showed enhanced calpain activity in lungs of LPS-challenged rats as indicated by a rise in apoptosis-inducing factor (AIF) in the nucleus and cleaved α-fodrin in the cytosol. These data imply that both caspase-dependent and caspase-independent routes may contribute to cell death associated with LPS-induced ALI. In lungs of LV_T and HV_T-ventilated mice, however, we only observed elevated levels of cleaved α-fodrin suggesting that 5 hours of mechanical ventilation primarily activates the caspase-independent cell death pathway (figure 2). In view of this notion, it would be of interest to evaluate the effects of calpain-inhibitors in our model of VILI. Cuzzocrea et al. showed that calpain-inhibitors may decrease the degree of lung injury and inflammation induced by zymosan, a polysaccharide from the yeast cell wall which leads to the formation of reactive oxygen species (ROS) [76]. More importantly, these authors demonstrated that calpain-inhibitors did not only reduce injury and inflammation in the lung but also in organs distal to the lung such as liver and intestine. As described in **chapter 2**, we observed that mechanical ventilation induces significant endothelial activation and pro-inflammation in both the lung and distal organs. Therefore, treatment with calpain-inhibitors may be beneficial in ventilated critically ill patients suffering from acute respiratory failure and/or MOF.

For many years, necrosis has been considered to be an uncontrolled process. Yet, recent evidence suggests that the course of necrotic cell death might be as tightly regulated as apoptotic cell death [77,78]. It has to be kept in mind though, that necrotic and apoptotic traits might co-exist [79] and that the same cell death inducers may promote either necrosis or apoptosis depending on the specific environmental setting [80]. In this regard, several investigators demonstrated that triggering of TNFR-1 by TNF-α does not only lead to activation of the extrinsic pathway of apoptosis but also to programmed necrosis involving receptor-interacting protein (RIP) kinases [80,81]. Necrostatin has been described as a small molecule inhibitor of programmed cell necrosis and may prevent RIP1 kinase activation [82] with possible anti-inflammatory effects [83]. Importantly, inhibition of programmed cell necrosis by necrostatin has been shown to protect against the development of brain injury [82-84]. Since mechanical ventilation in our model of VILI primarily activated the caspase-independent route of cell death, treatment with necrostatin may be considered as a potential therapeutic intervention strategy in ventilated critically ill patients.

The attenuation of caspase-independent cell death - via calpain-inhibitors or RIP-kinase inhibitors such as necrostatin - might be a preventive strategy for the devastating mechano-sensitive effects of mechanical ventilation and subsequently improve clinical outcome of

the critically ill patient. Considering the effects of stem cell treatment on restoring function of damaged tissue, it is tempting to speculate that autologous stem cell transplantation could become a beneficial approach to treat ventilated critically ill patients as well. Nonetheless, preventing prolonged exposure to mechanical (over)stretch by reducing tidal volumes remains of utmost importance.

Conclusions

1. Mechanical ventilation with high pressures does not only provoke endothelial activation and pro-inflammation in the lung but also induces a pro-inflammatory environment in organs distal to the lung (**chapter 2**). Moreover, HV_T-ventilation impairs functioning of peripheral lymphocytes (peripheral immune suppression; **chapter 7**).
2. FcγR-targeted IgG-Dex-liposomes are pharmacologically more effective than Dex-liposomes in particular with respect to inhibition of granulocyte influx induced by LV_T or HV_T-ventilation (**chapter 3**). Especially IgG-Dex-liposomes downregulate most parameters of ventilator-induced lung inflammation as efficiently as free dexamethasone.
3. Treatment with either dexamethasone or Ang-1 did not prevent the development of the more crude parameters of VILI (i.e. alveolar-capillary permeability, pulmonary edema, impaired gas exchange) despite inhibition of lung inflammation and VEGF expression (**chapters 4 and 5**). Thus, prevention of the pro-inflammatory response does not preclude loss of pulmonary function implying that lung inflammation and injury are two independent components of VILI.
4. LPS-induced ALI is associated with activation of both caspase-dependent and caspase-independent pathways of cell death, with a crucial role for p53 and calpains respectively (**chapter 6**). In contrast, 5 hours of LV_T or HV_T-ventilation primarily activated caspase-independent pathways of cell death (**chapter 7**).

SUMMARY

Although mechanical ventilation is a life-saving procedure in the intensive care unit, it may exacerbate or even initiate damage to the lung itself [1,2]. An intriguing clinical observation is that most critically ill ALI/ARDS patients do not succumb to acute lung failure but rather to progressive non-pulmonary organ dysfunction, so-called MOF [85-87]. Based on this notion, the first part of this thesis describes the effects of mechanical ventilation on the inflammatory response in healthy mice (**chapter 2**). We investigated whether these ventilator-induced effects occurred not only in the lung but also in organs distal to the lung. Previous studies revealed that the cyclic opening and collapse of alveoli during mechanical ventilation may provoke alveolar stretch and subsequently result in VILI [88,89]. To investigate the effects of alveolar stretch *in vivo*, we applied a ventilation strategy that has been described to cause overstretch of lung tissue [24,72]. We showed that mechanical ventilation increased the pro-inflammatory state of the lung but also of organs distal to the lung, like liver and kidney. These data indicate that alveolar stretch due to mechanical ventilation may play a significant role in the pathogenesis of MOF.

In the second part of this thesis, we evaluate the effects of different therapeutic intervention strategies on ventilator-induced lung inflammation and injury (**chapters 3, 4 and 5**). It has been thought that a ventilator-induced increase in pulmonary granulocytes, cytokines and chemokines may be crucially involved in the development of lung injury [36-38]. One of the most potent drugs to treat lung inflammation are glucocorticoids, which exert their effects by binding to intracellular glucocorticoid receptors (GRs) [90,91]. Although glucocorticoids successfully inhibit lung inflammation, systemic administration may lead to severe side effects such as increased blood glucose levels [44,45]. Therefore, we studied whether liposome-encapsulated dexamethasone may diminish lung inflammation induced by 5 hours of low (LV_T) or high tidal volume (HV_T)-ventilation (**chapter 3**). We hypothesized that targeting of FcγRs on e.g. activated granulocytes, by conjugating IgG to the Dex-liposomes, may enhance the efficacy of these liposomes in attenuating granulocyte influx, one of the hallmarks of VILI. The major finding was that FcγR-targeted IgG-Dex-liposomes are pharmacologically more effective than Dex-liposomes in particular with respect to inhibition of granulocyte infiltration induced by LV_T or HV_T-ventilation. Especially IgG-Dex-liposomes downregulate most parameters of ventilator-induced lung inflammation as efficiently as free dexamethasone. Next, we evaluated whether the anti-inflammatory action of dexamethasone protects ventilated mice against vascular leakage and impaired gas exchange (**chapter 4**). Interestingly, we observed that dexamethasone administration did not prevent the increase in BALf protein level, the increase in pulmonary wet-to-dry ratio and the reduction in PaO_2/FiO_2 ratio induced by mechanical ventilation even though it completely abolished pulmonary inflammation.

Loss of integrity of epithelial and endothelial cell monolayers has been suggested to play an important role in the ventilator-induced disruption of the alveolar-capillary barrier [28]. The Ang-Tie2 system regulates vascular integrity and has therefore been proposed to be crucially involved in the pathogenesis of pulmonary diseases like ALI/ARDS. Since vascular leakage and inflammation are important features of VILI, we hypothesized that Ang-Tie2 signaling plays a (protective) role in the development of VILI (**chapter 5**). We found that especially HV_T-ventilation influenced the Ang-Tie2 system implying that alterations in the Ang-Tie2 system are involved in the pathogenesis of VILI. Next, we evaluated whether treatment with Ang-1 (Tie2 receptor agonist) would protect against inflammation, vascular leakage and impaired gas exchange induced by HV_T-ventilation. The major finding of this study was that treatment with Ang-1 affects only specific aspects of VILI. We observed that Ang-1 administration markedly attenuated infiltration of granulocytes and expression of inflammatory mediators, VEGF and Ang-2 (Tie2 receptor antagonist) in lungs of HV_T-ventilated mice. Nonetheless, Ang-1 administration did not prevent vascular leakage and impaired gas exchange, the more crude parameters of VILI.

Based on our dexamethasone and Ang-1 intervention studies, we would like to suggest that treatment with anti-inflammatory agents does not prevent the aspects of VILI driven by mechanosensitive alterations in barrier properties [52], such as vascular leakage and impaired gas exchange, but will only regulate the pulmonary inflammation.

The third part of this thesis describes in more detail one of the possible mechanisms underlying the development of lung injury. Pulmonary cell death is thought to be involved in the ventilator-induced disruption of alveolar-capillary barriers [28]. Therefore, we determined which routes of cell death may be activated during lung injury. We investigated whether mechanical ventilation may initiate caspase-dependent and/or caspase-independent pathways of cell death (**chapter 7**). Our data show unequivocally that mechanical ventilation does not influence basal cleavage of caspase-8 nor that it induces cleavage of caspase-9 and -3. Levels of cleaved α-fodrin, however, were markedly increased after LV_T or HV_T-ventilation. To investigate whether caspase-dependent and/or caspase-independent routes of cell death were activated during endotoxin-induced lung inflammation, we used an *in vivo* rat model of LPS-induced ALI (**chapter 6**). We observed that LPS-induced lung injury was characterized by early activation of the extrinsic apoptotic pathway and late activation of the intrinsic apoptotic pathway accompanied by increased expression of p53 (important mediator of both intrinsic and extrinsic apoptotic pathways). Moreover, we demonstrated that LPS instillation in the lung activated caspase-independent cell death pathways as we found an increase in AIF in the nucleus and cleaved α-fodrin in the cytosol. In conclusion, LPS-challenge provokes lung injury via caspase-dependent apoptosis and caspase-independent necrosis, whereas lung injury induced by mechanical ventilation is for the greater part caspase-independent and probably executed by pathways involving a destabilization of the cytoskeleton via proteolytic activity of calpains.

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CHAPTER

8

Nederlandse samenvatting (Summary in Dutch)

Kunstmatige beademing is een standaard procedure op de intensive care afdeling en wordt toegepast om de ademhaling van zieke patiënten te ondersteunen. Vooral ernstige longaandoeningen vereisen kunstmatige beademing, zoals acute lung injury (ALI) of het acute respiratory distress syndrome (ARDS). In Nederland worden jaarlijks zo'n 8000 ALI/ARDS patiënten opgenomen die kunstmatige beademing behoeven. Ongeveer de helft van de volwassen ALI/ARDS patiënten overlijdt aan de gevolgen van deze ernstige longaandoeningen.

Hoofdstuk 1 beschrijft de huidige kennis over de effecten van kunstmatige beademing. Hoewel kunstmatige beademing een levensreddende procedure is, kan de overmatige rek van longweefsel op zichzelf schade veroorzaken aan zowel gezonde als zieke longen (ventilator-induced lung injury, VILI). Belangrijke kenmerken van VILI zijn een ontstekingsreactie, verhoogde doorlaatbaarheid (permeabiliteit) van het alveolaire-capillaire membraan, opeenhoping van eiwitrijk vocht (oedeem) en een verstoorde gasuitwisseling. Sinds de introductie van kunstmatige beademing heeft men erkend dat de overmatige rek van longweefsel het resultaat kan zijn van grote volumes lucht in de long, maar ook van het herhaaldelijk openen en samenvallen van longblaasjes (alveoli). Helaas kunnen hoge drukken of grote teugvolumes niet altijd worden vermeden tijdens de kunstmatige beademing van ernstig zieke patiënten met relatief stugge, beschadigde longen.

Onlangs heeft men aangetoond dat VILI niet alleen veroorzaakt wordt door "longbeschadigende" beademingsstrategieën met hoge drukken of grote teugvolumes, maar ook door "longbeschermende" beademingsstrategieën met lage drukken of kleine teugvolumes. Naast de verbetering van huidige beademingsstrategieën zijn dus aanvullende therapeutische interventies nodig om zieke patiënten te beschermen tegen de schadelijke effecten van kunstmatige beademing. De belangrijkste focus van dit proefschrift is te evalueren of verschillende therapeutische interventies bescherming kunnen bieden tegen verscheidene aspecten van VILI zoals de ontstekingsreactie, permeabiliteit van het alveolaire-capillaire membraan en een verstoorde gasuitwisseling.

KUNSTMATIGE BEADEMING: LOKALE EN SYSTEMISCHE EFFECTEN

Een intrigerende klinische observatie is dat de meeste beademde ALI/ARDS patiënten niet overlijden aan acuut longfalen, maar aan multi-orgaanfalen (multiple organ failure, MOF). Het is dan ook van groot belang inzicht te krijgen in de schadelijke effecten van kunstmatige beademing op de organen distaal van de long. Onderzoek heeft uitgewezen dat interacties tussen witte bloedcellen (leukocyten) en endotheelcellen een belangrijke rol spelen in het ontstaan van ernstige longziekten gerelateerd aan VILI, zoals ALI/ARDS, maar ook in het ontstaan van MOF. Om deze reden hebben we in **hoofdstuk 2** onderzocht of de overmatige rek van longweefsel door kunstmatige beademing leidt tot de activatie van endotheelcellen,

niet alleen in de long maar ook in organen distaal van de long (zoals lever en nieren). Om de effecten van kunstmatige beademing te bestuderen, hebben we gebruik gemaakt van een experimenteel model voor VILI in gezonde muizen. We hebben laten zien dat 4 uur beademing met hoge drukken resulteert in de activatie van endotheelcellen in de long maar ook in de lever en nieren. Geactiveerde endotheelcellen produceren ontstekingsmediatoren (cytokinen/chemokinen) en brengen meer adhesiemoleculen op hun celoppervlak tot expressie, waardoor leukocyten vanuit de bloedbaan de organen kunnen binnen komen. Een overvloed aan leukocyten in aangedaan weefsel kan uiteindelijk resulteren in weefselschade. Aangezien wij hebben aangetoond dat endotheelactivatie na kunstmatige beademing geassocieerd is met een verhoogd aantal leukocyten in zowel de long als de lever en nieren, lijkt het aannemelijk dat kunstmatige beademing een rol speelt in het ontstaan van MOF. Bovendien suggereert ons onderzoek in gezonde dieren dat een bestaande ontsteking geen voorwaarde is voor het optreden van de schadelijke effecten van kunstmatige beademing in distale organen. Niettemin, zal verder onderzoek nodig zijn om vast te stellen welke mechanismen ten grondslag liggen aan het ontstaan van een ontstekingsreactie in distale organen. Eerdere studies suggereren dat de verhoogde permeabiliteit van het alveolaire-capillaire membraan tijdens kunstmatige beademing leidt tot het vrijkomen van ontstekingsmediatoren in de systemische circulatie, welke vervolgens distale organen kunnen beïnvloeden. Wij laten echter zien dat beademingsgeïnduceerde effecten in de longen, lever en nieren gelijktijdig plaatsvinden. Dit impliceert dat het vrijkomen van ontstekingsmediatoren in de systemische circulatie waarschijnlijk niet de enige oorzaak is van het ontstaan van de ontstekingsreactie in distale organen. We kunnen echter niet uitsluiten dat de vrijgekomen ontstekingsmediatoren bijdragen aan *de novo* synthese van adhesiemoleculen, cytokinen en chemokinen in distale organen.

Naast een ontstekingsreactie in distale organen is er gesuggereerd dat de onderdrukking van het perifere immuunsysteem een belangrijke rol speelt bij de ontwikkeling van MOF. Aanvankelijk zal de onderdrukking van het perifere immuunsysteem een aanpassing zijn op de lokale ontsteking in de long, ter voorkoming en uitbreiding van de ontstekingsreactie en eventuele schade aan distale organen. Dit compensatiemechanisme kan maladaptief worden wanneer het langdurig aanhoudt, resulterend in overmatige onderdrukking van het perifere immuunsysteem en verhoogde vatbaarheid voor infecties. Eerder hebben we klinisch en experimenteel aangetoond dat de immuunfunctie van perifere leukocyten wordt onderdrukt door kunstmatige beademing. Voorlopige resultaten in ons muizenmodel voor VILI ondersteunen deze eerdere bevindingen (**hoofdstuk 7**). Als maat voor perifere immuunfunctie bepaalden we de mitogeen-geïnduceerde proliferatie van miltcellen (splenocyten). Wij hebben laten zien dat 5 uur beademing met hoge teugvolumes leidt tot een verlaging van de mitogeen-geïnduceerde proliferatie van splenocyten en dus tot een onderdrukking van het perifere immuunsysteem.

Onze resultaten tonen aan dat kunstmatige beademing niet alleen een ontstekingsreactie in distale organen (zoals de lever en nieren) veroorzaakt, maar ook de functie van het perifere immuunsysteem onderdrukt. De onderdrukte immuunfunctie van perifere leukocyten tijdens kunstmatige beademing van zieke patiënten zou kunnen leiden tot verhoogde vatbaarheid voor infecties en dus bijdragen aan het ontstaan van MOF.

THERAPEUTISCHE INTERVENTIES TER VOORKOMEN VAN VILI

In het tweede deel van dit proefschrift, evalueren we de effecten van verschillende therapeutische interventies op het ontstaan van VILI (**hoofdstukken 3, 4 en 5**). We hebben vooral gekeken of deze therapeutische interventies bescherming kunnen bieden tegen verscheidene aspecten van VILI zoals de ontstekingsreactie, verhoogde alveolaire-capillaire permeabiliteit en verstoorde gasuitwisseling. Ook bij het beantwoorden van deze vraagstellingen hebben we gebruik gemaakt van een experimenteel model voor VILI in gezonde muizen.

Effect van dexamethason op de ontstekingsreactie ten gevolge van kunstmatige beademing

Het besef dat leukocyten en ontstekingsmediatoren kunnen bijdragen aan het ontstaan van ernstige longziekten heeft geresulteerd in het gebruik van ontstekingsremmers. Dexamethason is een synthetisch glucocorticoid en een veelgebruikte ontstekingsremmer op de intensive care. Hoewel dexamethason succesvol is in het remmen van een ontstekingsreactie, kan behandeling met deze ontstekingsremmer leiden tot ernstige bijwerkingen als verhoogde bloedsuikerspiegels. Eerder onderzoek heeft aangetoond dat deze bijwerkingen beperkt kunnen worden wanneer dexamethason wordt verpakt in liposomen. In **hoofdstuk 3** hebben wij bestudeerd of liposoom-verpakt dexamethason (Dex-liposomen) de ontstekingsreactie in longen van beademde muizen kan verminderen. Omdat geactiveerde leukocyten (in het bijzonder, granulocyten) een belangrijke rol spelen bij het ontstaan van VILI, veronderstelden we dat het stimuleren van de opname van Dex-liposomen door *geactiveerde* immuuncellen de werkzaamheid van deze liposomen in ons model voor VILI zou verbeteren. Om Dex-liposomen te targeten naar Fcγ-receptoren (FcγRs) op geactiveerde macrofagen en granulocyten, hebben wij IgG aan Dex-liposomen gekoppeld. Onze hypothese was dat koppeling van IgG aan Dex-liposomen de binding van het IgG Fc-fragment op Dex-liposomen met de FcγRs op macrofagen en granulocyten zal bevorderen, en dus ook de effecten van Dex-liposomen in deze immuuncellen. De belangrijkste bevinding was dat IgG-Dex-liposomen farmacologisch effectiever zijn dan Dex-liposomen, voornamelijk met betrekking tot het voorkomen van de granulocyten infiltratie in longen van beademde muizen. Met name IgG-Dex-liposomen remmen de meeste parameters van de beademingsgeïnduceerde ontstekingsreactie even

efficiënt als vrij dexamethason. Liposoom-verpakt dexamethason heeft echter als voordeel dat het lokaal wordt vrijgegeven en dus de niet-gewenste bijwerkingen kan voorkomen.

Effect van dexamethason op de longschade ten gevolge van kunstmatige beademing

Er wordt gesuggereerd dat de beademingsgeïnduceerde ontstekingsreactie in de long kan leiden tot een verminderde longfunctie. In **hoofdstukken 3 en 4** hebben wij laten zien dat beademing met kleine (LV_T) of grote teugvolumes (HV_T) resulteert in verhoogde productie van ontstekingsmediatoren ten opzichte van niet-beademde dieren, wat gepaard gaat met een infiltratie van granulocyten. Het aantal granulocyten in de broncho-alveolaire lavage (wassing van alveoli) en de expressie van ontstekingsmediatoren waren zelfs verhoogd wanneer de longen van HV_T-beademde muizen werden vergeleken met die van LV_T-beademde muizen (**hoofdstuk 4**). Omdat een verstoorde gasuitwisseling alleen werd waargenomen na 5 uur HV_T-beademing, lijkt het aannemelijk dat de ontstekingsreactie in de long uiteindelijk een verminderde longfunctie tot gevolg heeft. In **hoofdstuk 4** hebben we daarom gekeken of de ontstekingsremmende werking van dexamethason het ontstaan van de alveolaire-capillaire permeabiliteit en verstoorde gasuitwisseling door kunstmatige beademing kan voorkomen dan wel beperken. In overeenstemming met eerdere studies hebben wij aangetoond dat behandeling met dexamethason de ontstekingsreactie ten gevolge van kunstmatige beademing compleet voorkomt. Bovendien verminderde dexamethason de expressie van vascular endothelial growth factor (VEGF, induceert vaatlekkage) in de longen van LV_T en HV_T-beademde muizen. Ondanks de remming van de ontstekingsreactie en VEGF expressie beschermt de behandeling met dexamethason niet tegen het ontstaan van andere parameters van VILI, zoals de vorming van eiwitrijk oedeem en verstoring van de gasuitwisseling. In een eerdere studie heeft men beschreven dat de effecten veroorzaakt door de overmatige rek van longweefsel voor een groot deel bijdragen aan beademingsgeïnduceerde longschade. Aangezien de muizen in ons VILI model blootgesteld werden aan 5 uur kunstmatige beademing, lijkt het erop dat de progressieve longschade veroorzaakt door de langdurige rek van longweefsel niet kan worden beïnvloed door de ontstekingsremmende werking van dexamethason.

Effect van angiopoietine (Ang)-1 op de longschade ten gevolge van kunstmatige beademing

De primaire plaats waar gasuitwisseling in de alveoli plaatsvindt is het < 0,2 µm dunne alveolaire-capillaire membraan, bestaande uit alveolaire epitheelcellen en capillaire endotheelcellen. Al in de jaren 80 hebben onderzoekers laten zien dat kunstmatige beademing, en dus de overmatige rek van longweefsel, schade kan aanrichten aan het alveolaire-capillaire

membraan en uiteindelijk kan leiden tot een verstoorde gasuitwisseling. Naast celdood, zou het verlies van cel integriteit belangrijk kunnen zijn voor de beschadiging van het alveolaire-capillaire membraan tijdens kunstmatige beademing. Eén van de cruciale systemen die betrokken is bij de regulatie van endotheelcel integriteit is het Ang-Tie2 system. Continue activatie van de Tie2 receptor door Ang-1 (Tie2 receptor agonist) is een vereiste voor het in stand houden van de integriteit van de endotheelcel. Eerder klinisch en experimenteel onderzoek heeft aangetoond dat het Ang-Tie2 systeem een essentiële rol speelt in de ontwikkeling van longziekten gerelateerd aan VILI, zoals ALI/ARDS. Er is gesuggereerd dat veranderingen in het evenwicht tussen VEGF (pro-lekkage) en Ang-1 (anti-lekkage) vaatlekkage kunnen veroorzaken in een experimenteel model voor ALI. Naar aanleiding van deze bevindingen, hebben we de rol van het Ang-Tie2 systeem bestudeerd in ons experimenteel model voor VILI (**hoofdstuk 5**). Vooral in de longen van HV_T-beademde muizen hebben wij duidelijke veranderingen waargenomen in het Ang-Tie2 systeem. We hebben aangetoond dat de mRNA expressie van Tie2, Ang-1 en Ang-2 (Tie2 receptor antagonist) significant is verminderd in HV_T-beademde muizen ten opzichte van niet-beademde muizen. Op dit tijdstip, was er ook een trend tot verlaging zichtbaar in de eiwit expressie van Ang-1 en Ang-2. HV_T-beademing leidt daarnaast tot een verhoogde eiwit expressie van VEGF. Onze resultaten lijken erop te wijzen dat veranderingen in het Ang-Tie2 systeem, samen met een verhoogde VEGF expressie, betrokken zijn bij de ontwikkeling van longschade tijdens kunstmatige beademing.

Het Ang-Tie2 systeem wordt gezien als een mogelijk therapeutisch target in ernstige longziekten gerelateerd aan VILI, zoals ALI/ARDS. Om deze reden hebben we onderzocht of behandeling met Ang-1 bescherming kan bieden tegen de verhoogde alveolaire-capillaire permeabiliteit en verstoring van de gasuitwisseling veroorzaakt door kunstmatige beademing. We hebben aangetoond dat Ang-1 de infiltratie van granulocyten en productie van ontstekingsmediatoren in longen van HV_T-beademde muizen kan beperken. Daarnaast hebben we laten zien dat Ang-1 de stijging in VEGF eiwit expressie voorkomt. Ondanks de aanzienlijke vermindering van de ontstekingsreactie en VEGF expressie in de long, biedt behandeling met Ang-1 geen bescherming tegen de vorming van eiwitrijk oedeem en verstoring van de gasuitwisseling (de meer "ruwe" parameters van VILI). In tegenstelling tot ons onderzoek, zijn er beschermende effecten van Ang-1 op vaatlekkage aangetoond in experimentele modellen voor ALI. In deze modellen gebruikt men lipopolysaccharide (LPS, bestanddeel van buitenmembraan van gram-negatieve bacteriën) voor de inductie van longschade. Een verklaring voor dit verschil zou kunnen zijn dat een ontstekingsreactie niet de primaire oorzaak van longschade is in ons VILI model, zoals dat wel het geval is bij longschade veroorzaakt door LPS. Longschade als gevolg van (overmatige) rek van longweefsel zal waarschijnlijk niet kunnen worden hersteld door behandeling met Ang-1.

Hoewel dexamethason en Ang-1 potentiële kandidaten zijn om de ontstekingsreactie ten gevolge van kunstmatige beademing te beperken, suggereren onze resultaten dat deze therapeutische interventies minder geschikt zijn om de meer "ruwe" parameters van VILI te beïnvloeden, zoals de vorming van eiwitrijk oedeem en verstoring van de gasuitwisseling. De bevindingen beschreven in dit proefschrift hebben een geheel nieuw licht geworpen op de vermeende mechanismen die ten grondslag liggen aan het ontstaan van VILI en wij achten deze van groot belang voor het definiëren van betere therapeutische strategieën in beademde ALI/ARDS patiënten.

INTERLEUKINE (IL)-6: MOGELIJKE ROL IN HET ONTSTAAN VAN VILI

Zowel klinische als experimentele studies beschrijven dat kunstmatige beademing de productie van de ontstekingscytokine IL-6 kan veroorzaken. In **hoofdstukken 3, 4 en 5** hebben wij laten zien dat ook in ons experimenteel VILI model sprake is van sterk verhoogde IL-6 productie, vooral in longen van HV_T-beademde muizen. Een interessante bevinding was dat IgG-Dex-liposomen en Dex-liposomen de IL-6 productie veroorzaakt door HV_T-beademing niet kon beïnvloeden, terwijl deze liposomen de IL-1β productie volledig kon remmen (**hoofdstuk 3**). De resultaten lijken erop te wijzen dat de target cellen van de gebruikte liposomen, voornamelijk geactiveerde macrofagen en granulocyten, niet de enige bron van IL-6 productie zijn. Het is bekend dat IL-6 kan worden gemaakt door een breed scala van longcellen. *In vitro* studies hebben laten zien dat alveolaire epitheelcellen en capillaire endotheelcellen ook geactiveerd worden door overmatige rek, waardoor ontstekingsmediatoren terechtkomen in omliggend longweefsel. Aangezien de IL-6 expressie na behandeling met IgG-Dex-liposomen of Dex-liposomen hoog bleef, is het verleidelijk om te speculeren dat de epitheel- en endotheelcellen deze liposomen niet opnemen en dus IL-6 kunnen blijven produceren. Het feit dat zowel IgG-Dex-liposomen als Dex-liposomen effectief de IL-1β en keratinocyte-derived chemokine (KC) expressie kunnen remmen, welke voornamelijk worden gemaakt door alveolaire macrofagen, ondersteunt deze hypothese.

Tijdens een ontstekingsreactie spelen geactiveerde endotheelcellen een cruciale rol bij het uittreden van leukocyten uit de bloedbaan, o.a. via de expressie van adhesiemoleculen (als E-selectine) op het celoppervlak. In **hoofdstuk 2** hebben we aangetoond dat kunstmatige beademing resulteert in een verhoogde expressie van E-selectine in het longweefsel, wat gepaard ging met een infiltratie van granulocyten. Om Dex-liposomen te targeten naar geactiveerd endotheel, hebben wij een antilichaam tegen E-selectine aan Dex-liposomen gekoppeld (immunoliposomen). In tegenstelling tot IgG-Dex-liposomen, waren de immunoliposomen wel in staat de beademingsgeïnduceerde IL-6 expressie te verminderen (**hoofdstuk 7**). Het zou dan ook goed mogelijk kunnen zijn dat de endotheelcellen in de long de voornaamste bron van IL-6 productie zijn tijdens kunstmatige beademing. Verder onderzoek

is nodig om vast te stellen waarom Ang-1 behandeling niet leidt tot een vermindering van IL-6 expressie, terwijl Ang-1 zich primair richt op endotheelcellen (**hoofdstuk 5**).

Uit ons onderzoek blijkt dat IL-6 minder goed reageert op de behandeling met dexamethason of Ang-1 dan andere ontstekingsmediatoren (als IL-1β). Onze data suggereren dat IL-6 een cruciale rol speelt bij het ontstaan van longschade tijdens kunstmatige beademing. Voor toekomstige therapeutische interventies is het van belang om te bepalen welke cellen IL-6 produceren tijdens de ontwikkeling van VILI.

TOEKOMSTIGE THERAPEUTISCHE INTERVENTIES IN ERNSTIG ZIEKE PATIËNTEN MET VILI

Onlangs heeft men beschreven dat zelfs "longbeschermende" beademingsstrategieën de ontwikkeling van belangrijke parameters van VILI kunnen veroorzaken. Onze resultaten uit **hoofdstukken 3 en 4** bevestigen deze eerdere bevindingen. In ons experimenteel VILI model hebben we waargenomen dat niet alleen de conventionele beademingstrategieën met grote teugvolumes (HV_T-beademing) maar ook de klinisch relevante beademingsstrategieën met kleine teugvolumes (LV_T-beademing) leidt tot een ontstekingsreactie en vaatlekkage in de long. Het gebruik van aanvullende therapeutische interventies zal bij kunnen dragen aan de overleving van beademde patiënten op de intensive care afdeling.

In **hoofdstukken 4 en 5** hebben we laten zien dat het ontstaan van de meer "ruwe" parameters van VILI niet kan worden voorkomen door het remmen van de ontstekingsreactie in de long. Gezien deze resultaten, willen wij stellen dat ontstekingsremmers minder geschikt zijn om longschade door de overmatige rek van longweefsel te bestrijden. Niettemin kan men ontstekingsremmende therapeutische interventies overwegen wanneer een ontsteking de primaire oorzaak is van longschade, zoals in niet-beademde patiënten met ALI/ARDS.

De bevindingen in dit proefschrift suggereren dat de toekomstige therapeutische interventies in beademde patiënten zich moeten richten op het voorkomen of beperken van schade aan het alveolaire-capillaire membraan. Naast het verlies van cel integriteit, is het bekend dat celdood een essentiële rol speelt in de beschadiging van het alveolaire-capillaire membraan tijdens kunstmatige beademing. In een *in vitro* model heeft men waargenomen dat de overmatige rek van longweefsel verschillende routes van celdood kan activeren, i.e. apoptose en necrose. Op basis hiervan, hebben wij gekeken welke routes van celdood worden geactiveerd in ons experimentele model voor VILI (**hoofdstuk 7**). Als een maat voor caspase-afhankelijke celdood (apoptose) hebben we de eiwit expressie van actief caspase-8, -9 en -3 bepaald in longen van beademde muizen. Onze resultaten laten zien dat de basale expressie van actief caspase-8 niet wordt beïnvloed door LV_T of HV_T-beademing. Daarnaast was de eiwit expressie

van actief caspase-9 en -3 beneden de detectielimiet in alle experimentele groepen, wat impliceert dat caspase-afhankelijke apoptotische celdood routes niet worden geactiveerd door 5 uur kunstmatige beademing. Verhoogde niveaus van intracellulair calcium kunnen resulteren in de activatie van verschillende eiwitten waaronder calpaines. Geactiveerde calpaines zorgen voor de enzymatische splitsing van het cytoskelet eiwit α-fodrine, wat vervolgens kan leiden tot de destabilisatie van cellen en uiteindelijk tot caspase-onafhankelijke necrotische celdood. We hebben laten zien dat de eiwit expressie van α-fodrine in longen van LV_T en HV_T-beademde muizen significant verhoogd was vergeleken met die van niet-beademde muizen. Onze resultaten lijken er op te wijzen dat beademingsgeïnduceerde celdood met name wordt veroorzaakt door caspase-onafhankelijke necrotische routes. Omdat eerder onderzoek in een vergelijkbaar VILI model heeft aangetoond dat kunstmatige beademing resulteert in een stijging van het aantal caspase-3 positieve cellen, kunnen we niet kunnen uitsluiten dat caspase-afhankelijke apoptotische routes in geringe mate zijn geactiveerd.

In **hoofdstuk 6** hebben we onderzocht of de verschillende celdood routes geactiveerd worden in LPS-geïnduceerde ALI, een experimenteel model gerelateerd aan VILI. Het is bekend dat veel van de kenmerken van ALI nagebootst kunnen worden door het geven van LPS in de longen, waardoor het als een geschikt model voor acuut longfalen wordt beschouwd. We hebben aangetoond dat de eiwit expressie van actief caspase-8, -9 en -3 verhoogd wordt door LPS toediening, wat gepaard gaat met een verhoogde expressie van p53 (een belangrijke mediator van apoptose). Bovendien hebben we laten zien dat LPS de calpaine activiteit in longen van ratten stimuleert zoals aangegeven door een toename van apoptosis-inducing factor (AIF) in de kern en actief α-fodrine in het cytosol. Deze gegevens suggereren dat zowel caspase-afhankelijke als caspase-onafhankelijke routes bijdragen aan celdood tijdens LPS-geïnduceerde ALI.

Bovenstaande resultaten lijken erop te wijzen dat longschade door LPS-toediening wordt veroorzaakt door zowel caspase-afhankelijke als caspase-onafhankelijke celdood routes, terwijl longschade door kunstmatige beademing voornamelijk wordt veroorzaakt door caspase-onafhankelijke celdood routes. Het is dan ook verleidelijk om te speculeren dat het voorkomen van caspase-onafhankelijke celdood, door de toediening van bijvoorbeeld calpaine-remmers, een gunstige benadering zal zijn voor het behandelen van beademde patiënten. Niettemin, blijft het beperken van langdurige blootstelling aan de overmatige rek van longweefsel van het grootste belang.

CONCLUSIES

1. Kunstmatige beademing met hoge drukken veroorzaakt een ontstekingsreactie, niet alleen in de long maar ook in distale organen zoals de lever en nieren (**hoofdstuk 2**). Daarnaast onderdrukt HV_T-beademing de immuunfunctie van perifere lymfocyten (**hoofdstuk 7**).
2. FcγR-gerichte IgG-Dex-liposomen zijn farmacologisch effectiever dan Dex-liposomen vooral met betrekking tot het beperken van granulocyten infiltratie in longen van beademde muizen (**hoofdstuk 3**). Met name IgG-Dex-liposomen remmen de meeste parameters van de beademingsgeïnduceerde ontstekingsreactie even efficiënt als vrij dexamethason.
3. Ondanks remming van de ontstekingsreactie en VEGF expressie beschermen dexamethason en Ang-1 niet tegen de ontwikkeling van de meer "ruwe" parameters van VILI zoals de verhoogde alveolaire-capillaire permeabiliteit, vorming van eiwitrijk oedeem en verstoorde gasuitwisseling (**hoofdstukken 4 en 5**). Het voorkomen van een ontstekingsreactie kan het verlies van de longfunctie niet tegengaan, wat impliceert dat longontsteking en -schade twee onafhankelijke componenten in de pathogonese van VILI zijn.
4. LPS-geïnduceerde ALI is geassocieerd met de activatie van caspase-afhankelijke en caspase-onafhankelijke celdood routes, met respectievelijk een rol voor p53 en calpaines (**hoofdstuk 6**). LV_T en HV_T-beademing zijn echter in de eerste plaats geassocieerd met caspase-onafhankelijke celdood routes (**hoofdstuk 7**).

LIST OF ABBREVIATIONS

ABIN	A20-binding inhibitor of NF-κB
AIF	Apoptosis-inducing factor
ALI	Acute lung injury
Ang	Angiopoietin
ANOVA	Analysis of variance
AP	Activator protein
APAF	Apoptotic protease-activating factor
ARDS	Acute respiratory distress syndrome
ATP	Adenosine triphosphate
BALf	Bronchoalveolar lavage fluid
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X protein
BCA	Bicinchoninic acid
Bcl	B-cell lymphoma
BE	Base excess
(t)-Bid	(truncated) BH-3 interacting domain death agonist
Bid$^{-/-}$	Bid homologous knockout
BSA	Bovine serum albumin
Ca^{2+}	Calcium
Calpain	Calcium-dependent cysteine protease
CARS	Compensatory anti-inflammatory response syndrome
Caspase	Cysteine aspartyl-specific protease
Cathepsin	Catabolic aspartyl protease
COX IV	Cytochrome *c* oxidase complex IV
CTAC	Cetyltrimethylammonium chloride
Dex-liposomes	Dexamethasone encapsulated in liposomes
DISC	Death-inducing signaling complex
(c)DNA	(complementary) Deoxyribonucleic acid
DTT	Dithiothreitol
EC	Endothelial cell
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
FADD	Fas-associated protein with death domain
FasL	Fas ligand
FcγR	Fcγ-receptor

FiO_2	Fractional inspired oxygen concentration
FOXO1	Forkhead transcription factor FKHR1
GAPdH	Glyceraldehyde 3-phosphate dehydrogenase
GR	Glucocorticoid receptor
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
H&E	Hematoxylin-eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HV_T	High tidal volume
ICAM	Intercellular adhesion molecule
ICU	Intensive care unit
IFN	Interferon
Ig	Immunoglobulin
IgG-Dex-liposomes	Dexamethasone encapsulated in IgG-modified liposomes
IL	Interleukin
Immunoliposomes	Dexamethasone encapsulated in anti-E-selectin modified liposomes
KC	Keratinocyte-derived chemokine
KCl	Potassium chloride
kDa	kilo Dalton
LPS	Lipopolysaccharide
LSD	Least significant difference
LV_T	Low tidal volume
MCP	Monocyte chemotatic protein
$MgCl_2$	Magnesium chloride
MIP	Macrophage inflammatory protein
MOF	Multiple organ failure
MPO	Myeloperoxidase
NaCl	Sodium chloride
NF-κB	Nuclear factor-kappa B
NVC	Non-ventilated controls
O_2	Oxygen
$PaCO_2$	Partial pressure of arterial carbon dioxide
p-Akt	Phospho-Akt
PaO_2	Partial pressure of arterial oxygen
PaO_2/FiO_2	Ratio of partial pressure arterial oxygen and fraction inspired oxygen
$p53^{-/-}$	p53 homologous knockout
PBS	Phosphate buffered saline
PECAM	Platelet-endothelial cell adhesion molecule
PEEP	Positive end-expiratory pressure

PI3-K	Phosphatidylinositol 3 kinase
PIP	Peak inspiratory pressure
PUMA	p53 upregulated modulator of apoptosis
P-V curve	Pressure-volume curve
RIP	Receptor-interacting protein
(m)RNA	(messenger) Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription-fpolymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electroforese
SEM	Standard error of the mean
SIRS	Systemic inflammatory response syndrome
Tie	Tyrosine kinase with Ig and EGF homology domains
TMB	3',5,5'-tetramethylbenzidine dihydrochloride
TNF	Tumor necrosis factor
TNFR	TNF receptor
TLR	Toll-like receptor
TLR4$^{-/-}$	TLR4 homologous knockout
Tris	Tris(hydroxymethyl)aminomethane
VCAM	Vascular cell adhesion molecule
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor
VILI	Ventilator-induced lung injury

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Van een schouderklopje heeft nog nooit iemand een blessure gekregen - Foppe de Haan

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Anne, Esther, Loes, Michaël, Moniek, Patricia, Paula, Stella, Vivianne, Xiyong en Zabi, jullie waren fijne collega's en ik heb mooie herinneringen aan jullie overgehouden.

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Jessica

List of publications

Hegeman MA, Hennus MP, van Meurs M, Cobelens PM, Kavelaars A, Jansen NJG, Schultz MJ, van Vught AJ, Molema G, Heijnen CJ. Angiopoietin-1 treatment reduces inflammation but does not prevent ventilator-induced lung injury. Submitted.

Hegeman MA, Hennus MP, Cobelens PM, Kavelaars A, Jansen NJG, Schultz MJ, van Vught AJ, Heijnen CJ. Downregulation of lung inflammation by dexamethasone does not prevent ventilator-induced lung injury. Submitted.

Hegeman MA, Cobelens PM, Kamps JAAM, Hennus MP, Jansen NJG, Schultz MJ, van Vught AJ, Molema G, Heijnen CJ. Liposome-encapsulated dexamethasone attenuates ventilator-induced lung inflammation: increased efficacy of Fcγ-receptor targeting. Submitted.

Cobelens PM, **Hegeman MA**, Kavelaars A, Jansen NJG, van Vught AJ, Kesecioglu J, Heijnen CJ. Activation of caspase-dependent and caspase-independent cell death after lipopolysaccharide-induced lung injury in rats. Submitted.

Hegeman MA, Hennus MP, Heijnen CJ, Specht PAC, Lachmann B, Jansen NJG, van Vught AJ, Cobelens PM. Ventilator-induced endothelial activation and inflammation in the lung and distal organs. Crit Care 2009, 13(6):R182

Tamashiro KLK, **Hegeman MA**, Nguyen MMN, Melhorn SJ, Ma LY, Woods SC, Sakai RR. Dynamic body weight and body composition changes in response to subordination stress. Physiol Behav 2007, 91(4): 440-448

Tamashiro KLK, **Hegeman MA**, Sakai RR. Chronic social stress in a changing dietary environment. Physiol Behav 2006, 89(4): 536-542

Curriculum Vitae

Jessica Hegeman werd geboren op 4 oktober 1981 te Hellendoorn. Zij behaalde het VWO diploma in 2000 aan het Pius X College te Almelo. In datzelfde jaar begon zij met de studie Biologie aan de Rijksuniversiteit Groningen te Groningen. Gedurende haar studie Biologie volgde zij twee wetenschappelijke stages. Haar eerste stage werd doorlopen op de afdeling Neuroendocrinologie van de Rijksuniversiteit Groningen onder begeleiding van Prof. Dr. Anton Scheurink en Dr. Céline Morens. Tijdens haar tweede stage deed zij onderzoek op het laboratorium voor Neuroendocrinologie van de Universiteit van Cincinnati te Cincinnati (Ohio, USA) onder begeleiding van Prof. Dr. Randall Sakai en Dr. Kellie Tamashiro. Het doctoraal examen van de opleiding Biologie (specialisatie Medische Biologie, variant Onderzoek) werd in december 2005 met veel genoegen behaald. In januari 2006 werd zij aangesteld als promovendus op een samenwerkingsproject tussen het laboratorium voor Neuroimmunology and Developmental Origins of Disease (onder supervisie van Prof. Dr. Cobi Heijnen en Dr. Pieter Cobelens) en de afdeling Intensive Care Kinderen (onder supervisie van Prof. Dr. Hans van Vught en Dr. Koos Jansen) van het Wilhelmina Kinderziekenhuis, onderdeel van het Universitair Medisch Centrum te Utrecht. Tevens verrichte zij als gastonderzoeker experimenten op het Laboratorium voor Experimentele Intensive Care en Anesthesiologie (Prof. Dr. Marcus Schultz) van het Academisch Medisch Centrum te Amsterdam en op de afdeling Anesthesiologie (Prof. Dr. Burkhard Lachmann) van de Erasmus Universiteit te Rotterdam. In de afgelopen 4,5 jaar deed zij onderzoek naar de mechanismen die mogelijk ten grondslag liggen aan het ontstaan van longschade en secundaire complicaties tijdens kunstmatige beademing van gezonde individuen. Daarnaast werd nagegaan of beademingsgeïnduceerde longschade voorkomen danwel beperkt kon worden door het toepassen van verschillende therapeutische interventies. De resultaten van het onderzoek staan in dit proefschrift beschreven. Op dit moment is zij werkzaam als post-doctoraal onderzoeker op het Laboratorium voor Experimentele Intensive Care en Anesthesiologie van het Academisch Medisch Centrum te Amsterdam, onder supervisie van Prof. Dr. Marcus Schultz.

Jessica Hegeman was born on October 4, 1981 in Hellendoorn, the Netherlands. She finished her secondary education at the Pius X College, Almelo in 2000. In the same year, she started to study Biology at the University of Groningen, Groningen. During her studies she attended two scientific internships. Her first internship was completed at the department of Neuroendocrinology of the University of Groningen under the guidance of Prof. Dr. Anton Scheurink and Dr. Céline Morens. During her second internship, she performed research at the laboratory of Neuroendocrinology of the University of Cincinnati, Cincinnati (Ohio, USA) under the guidance of Prof. Dr. Randall Sakai and Dr. Kellie Tamashiro. In December 2005, she graduated from the University of Groningen and was honoured a Master's Degree in Biology (with Medical Biology specialization and Research variant). In January 2006, she became a PhD-student and started to work on a collaborative project between the laboratory of Neuroimmunology and Developmental Origins of Disease (under supervision of Prof. Dr. Cobi Heijnen and Dr. Pieter Cobelens) and the department of Pediatric Intensive Care (under supervision of Prof. Dr. Hans van Vught and Dr. Koos Jansen) of the Wilhelmina Children's Hospital, part of the University Medical Center Utrecht. She also worked as a guest researcher at the Laboratory of Experimental Intensive Care and Anesthesiology (Prof. Dr. Marcus Schultz) of the Academic Medical Center, Amsterdam and at the department of Anesthesiology (Prof. Dr. Burkhard Lachmann) of the Erasmus University, Rotterdam. In the past 4.5 years, she studied the mechanisms that may underlie the development of lung injury and secondary complications during mechanical ventilation of healthy individuals. In addition, she evaluated whether ventilator-induced lung injury could be prevented or reduced by applying different therapeutic interventions. Results of these studies are described in this thesis. At present, she works as a post-doctoral researcher at the Laboratory of Experimental Intensive Care and Anesthesiology of the Academic Medical Center Amsterdam, under supervision of Prof. Dr. Marcus Schultz.

Color figures

CHAPTER 2

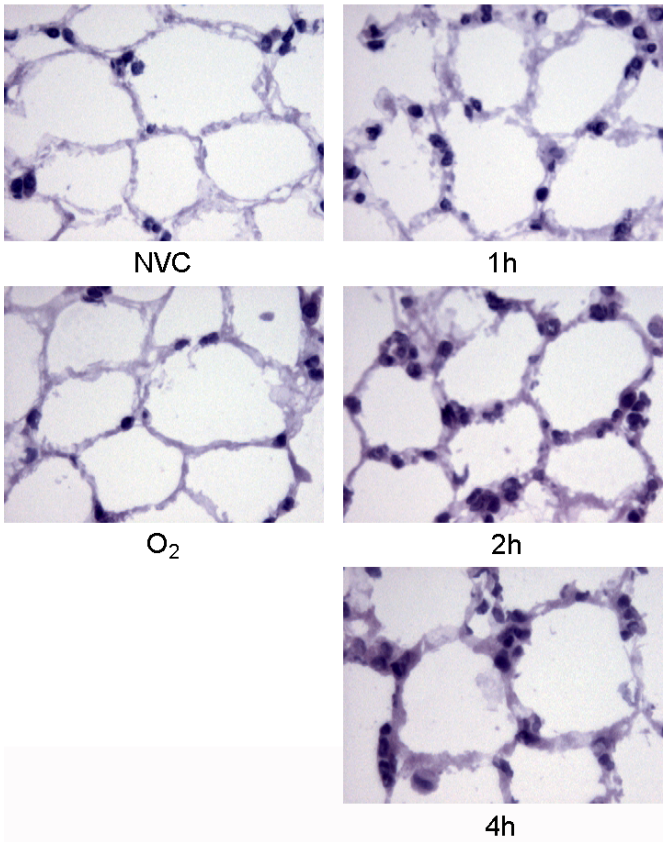

Figure 1: Histopathology of pulmonary tissue.
Lung sections were stained with hematoxylin-eosin (H&E) to analyze lung architecture and presence of granulocytes in pulmonary tissue. Magnification x500.
NVC = non-ventilated controls; 1h, 2h, 4h = mechanically ventilated for 1, 2 or 4 hours; O_2 = hyperoxia for 4 hours.

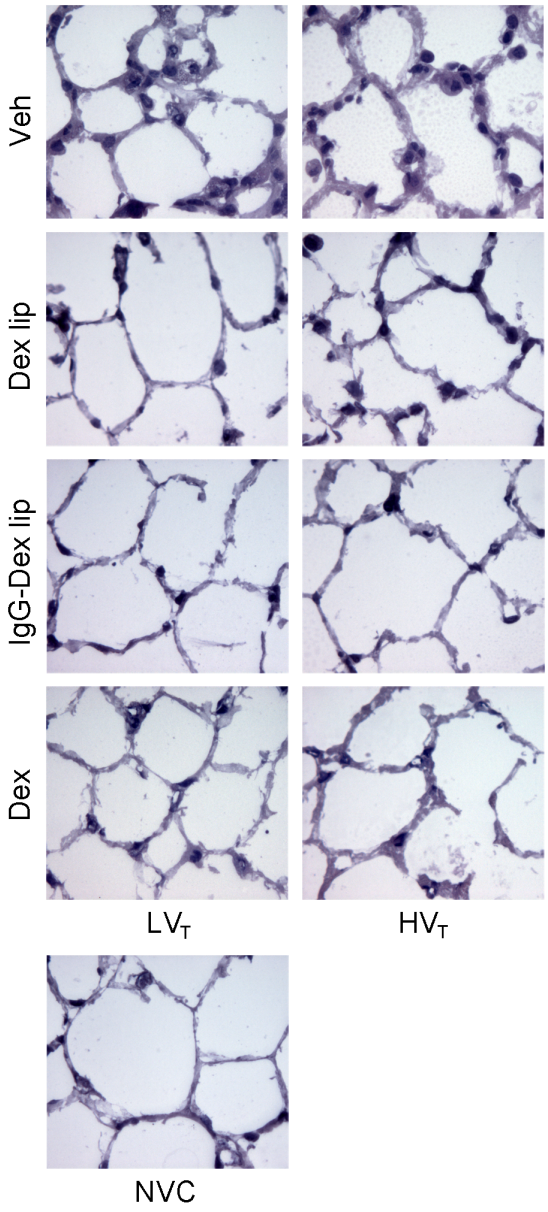

Figure 1: Effect of Dex-liposomes, IgG-Dex-liposomes and free dexamethasone on changes in lung histology induced by mechanical ventilation.

Lung sections were stained with hematoxylin-eosin (H&E) to analyze lung architecture and presence of granulocytes in pulmonary tissue. Magnification ×500.

NVC = non-ventilated controls; LV_T, HV_T = mechanically ventilated with low or high tidal volumes; Veh, Dex lip, IgG-Dex lip, Dex = intravenously treated with either vehicle (sterile saline), liposomes containing dexamethasone, IgG-modified liposomes containing dexamethasone, or free dexamethasone.

CHAPTER 4

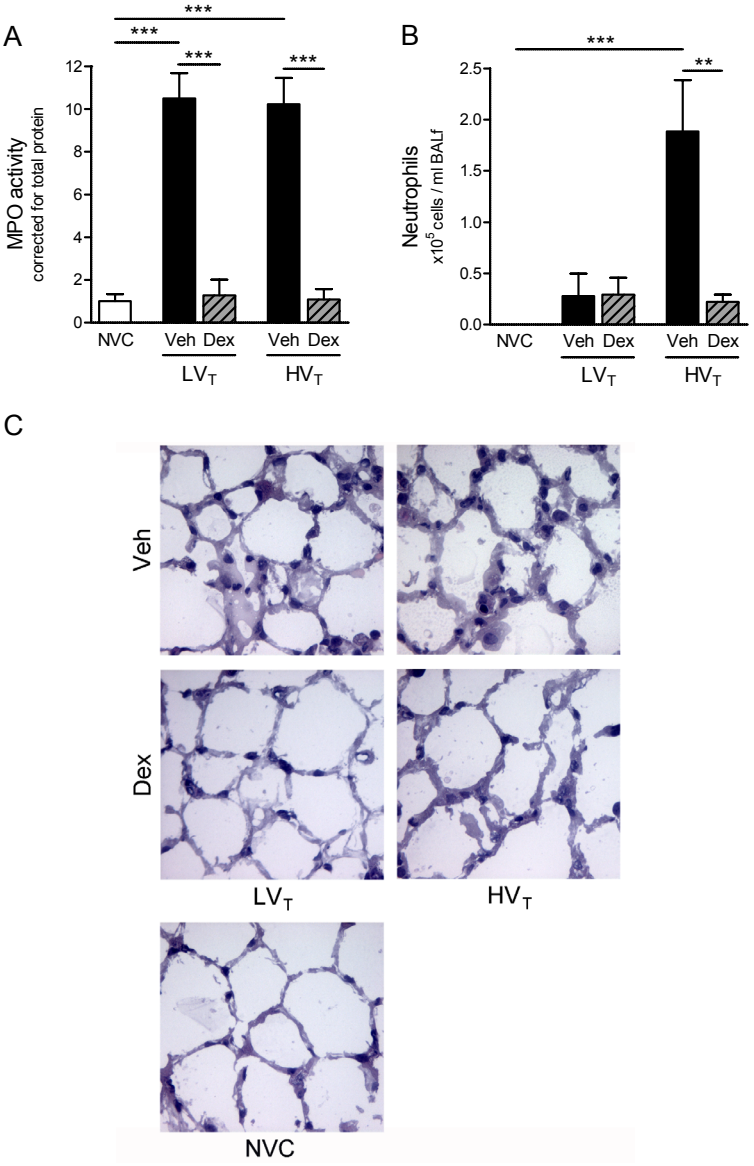

Figure 3: Dexamethasone treatment decreases infiltration of granulocytes.
A: In lung homogenates, myeloperoxidase (MPO) activity was determined as a measure of granulocyte influx. Levels were normalized for total protein levels. **B:** On bronchoalveolar lavage fluid (BALf) cytospin preparations, differential cell counts were done to determine neutrophil exudation into alveolar space. Data are expressed as mean ± SEM, and depicted relative to NVC. ** $p<0.01$, *** $p<0.001$. **C:** Lung sections were stained with hematoxylin-eosin (H&E) to analyze lung architecture and presence of granulocytes in lung tissue. Magnification ×500.
NVC = non-ventilated controls; LV_T, HV_T = mechanically ventilated with low or high tidal volumes; Veh, Dex = intravenously treated with either vehicle (sterile saline) or dexamethasone.

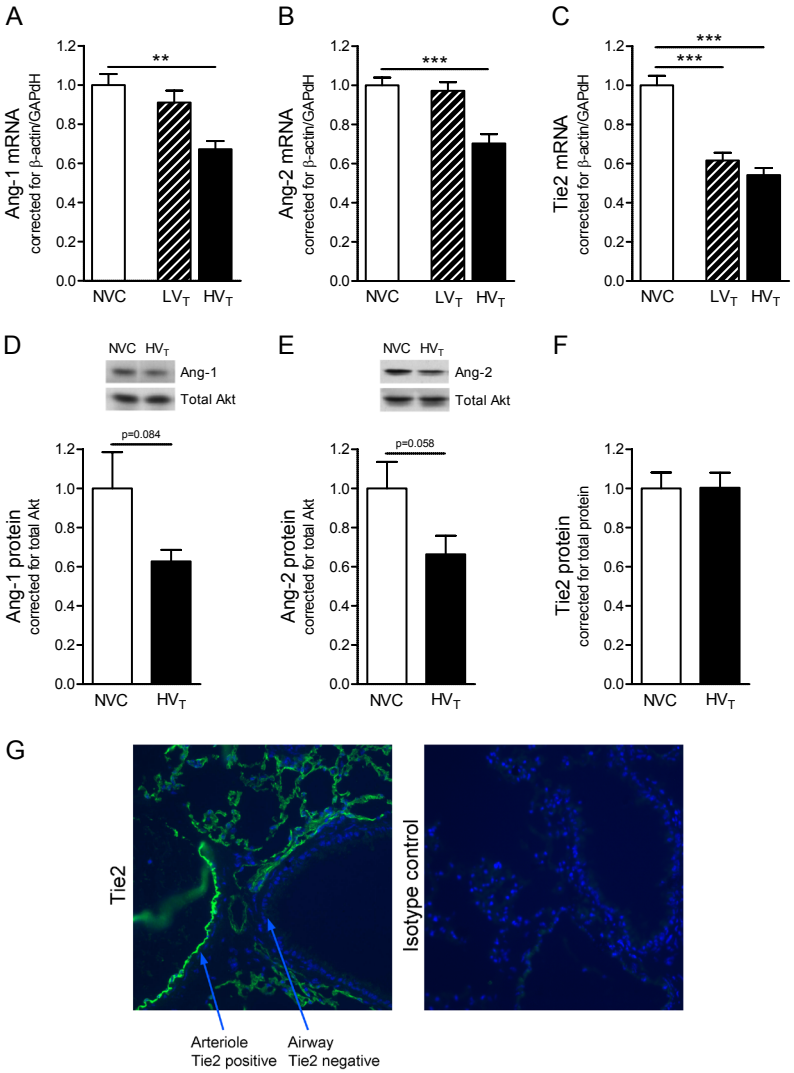

Figure 2: Mechanical ventilation affects the angiopoietin (Ang)-Tie2 system.
A-C: In lung homogenates, mRNA expression of Ang-1, Ang-2 and Tie2 was determined by real-time RT-PCR. Levels were normalized for expression of β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPdH). **D-E:** Protein expression of Ang-1 and Ang-2 was determined by Western blotting. Membranes were reprobed with antibody recognizing total Akt (Akt1/PKBα) to control for equal loading. Inset: representative Western blot depicting immunodetectable Ang-1 and Ang-2. **F:** Protein expression of Tie2 was determined by ELISA. Levels were normalized for total protein levels. Data are expressed as mean ± SEM, and depicted relative to NVC. ** $p<0.01$, *** $p<0.001$. **G:** Lung sections of NVC were stained with fluorescent antibody recognizing Tie2 to visualize the presence of Tie2 on pulmonary cells (isotype control was negative). Magnification ×200.

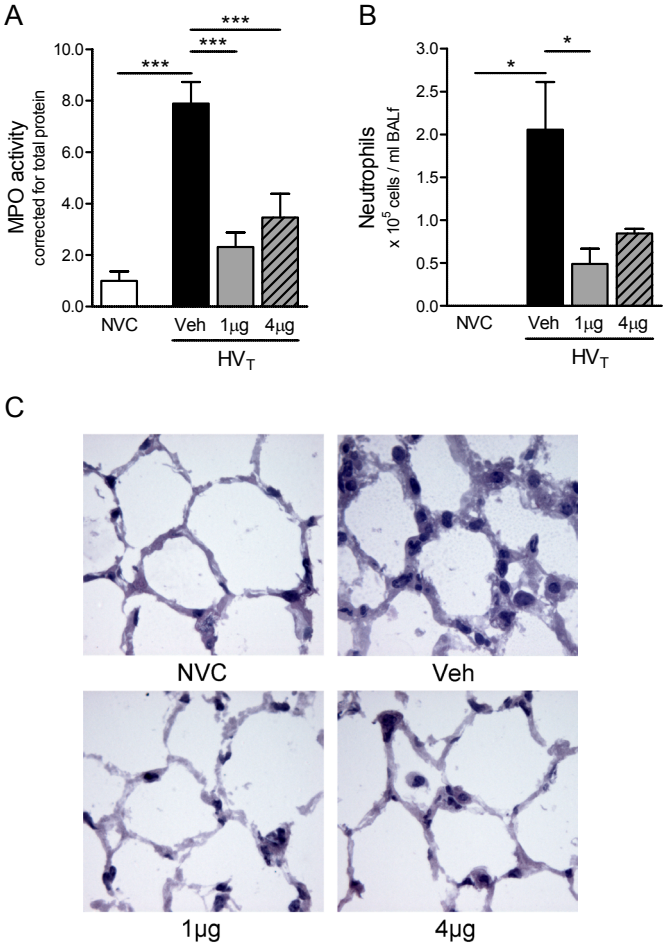

Figure 6: Angiopoietin (Ang)-1 treatment reduces granulocyte infiltration.
A: In lung homogenates, myeloperoxidase (MPO) activity was determined as a measure of granulocyte influx. Levels were normalized for total protein levels. **B:** On bronchoalveolar lavage fluid (BALf) cytospin preparations, differential cell counts were done to determine neutrophil exudation into alveolar space. Data are expressed as mean ± SEM, and depicted relative to NVC. * $p<0.05$, *** $p<0.001$. **C:** Lung sections were stained with hematoxylin-eosin (H&E) to analyze the presence of granulocytes in pulmonary tissue. Magnification ×500.
NVC = non-ventilated controls; LV_T, HV_T = ventilated with low or high tidal volumes; Veh, 1µg, 4µg = intravenously treated with either vehicle (saline), Ang-1 (1µg per animal), or Ang-1 (4µg per animal).