Doctoral dissertation

Carbon monoxide-mediated antiinflammatory effects in acute lung injury

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In memory of my grandfather

„E vers is valóság, akár az álmod.
Az élet: kés és szív egy szín alatt:
Szemeddel az egész tengert halászod
s horoggal mit fogsz? egynéhány halat.”
(Weöres Sándor)

“This poem is as real as are your dreams.
Life is both heart and knife, all of a kind.
Your eyes can land the whole sea in their nets.
What will you catch with a rod? A few stray sprats.”
(translation by George Szirtes)

“As long there is breath, there is hope.”
(a Nepali proverb)

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1.Abbreviations

ALI	-acute lung injury
AP-1	-activator protein-1
ARDS	-acute respiratory distress syndrome
CO	-carbon monoxide
HO	-heme oxygenase
IL	-interleukin
LPS	-lipopolysaccharide
MAPK	-mitogen-activated protein kinase
NFκB	-nuclear factor-κB
OV	-overventilation
PEEP	-positive end expiratory pressure
TNF	-tumor necrosis factor
VILI	-ventilator-induced lung injury

2.Introduction and specific aims

The need for understanding the mechanism of human diseases has always linked the basic scientist and the clinical physician. With the powerful new tools of molecular biology the human body can be explored to an extent like never before. However, in our globalized culture, when new diseases form and spread rapidly the need for joint efforts is the greatest. The combined work of molecular biology, population genetics, social sciences and clinical medicine can lead to a better description of human physiological and pathological response to the environment. A perfect example is the 2003 severe respiratory syndrome (SARS) epidemic where quick interdisciplinary collaboration brought the spread of the disease to a stop. Translational research is one of the most challenging new ways in which interdisciplinary medicine explores human pathology. The genetic analysis of health and disease can guide medicine to new therapeutic approaches precisely targeting the disease at a subcellular level. In the meantime, disease susceptibility, human racial differences, contributing environmental factors can be discovered. Translational research also opens up international collaboration opportunities as it shares tasks among scientists in many fields of interest.

As a practicing physician interested in basic science I was very happy to participate in a series of translational research projects about the beneficial effects of carbon monoxide on the human body. The respiratory system is one of the primary targets of most diseases. Lung injury is very common and because of its multifactoral etiology its mechanism is hardly understood. Our therapeutic solutions are very limited, mostly supportive. New ways of reducing acute lung damages is essential to

provide better care and extended life expectancy for patients with acute lung injury (ALI).

In my thesis work, I would like to explain the pathology of ALI and the beneficial effects of low dose inhaled CO with the help of two animal models I worked with in the past 3 years.

2.1 The history of Acute Lung Injury

Acute lung injury (ALI) became a highlighted topic of pulmonary medical care with the widespread availability of mechanical ventilation in the 1960`s. During the Vietnam War thousands of solders suffered multiple organ injuries and required mechanical ventilation. Lung injured patients could be kept alive for longer periods of time and weaned from ventilators leading to new dimensions of patient care and research. This is when acute respiratory distress syndrome, the most severe form of ALI was described by Ashbaugh and colleagues(1). It was first referred to as adult respiratory distress syndrome to distinguish the disease from the respiratory distress syndrome that occur in neonates. However today the more preferred name is acute respiratory distress syndrome (ARDS). Today ALI is one of the most investigated and debated field of pulmonary-intensive care medicine. With new therapeutic modalities and mechanical ventilator setting patients who were previously prone to death are given a chance to return to life.

A milestone in the history of ALI/ARDS is the ARDS Network Trials. The original trial collected patients from 1996 to 1999 in 10 University Medical Centers in the USA. Patients with mechanical ventilation and ALI, determined according to the American-European Consensus Conference guidelines (see criteria in Definitions),

were eligible to enroll the study. 861 patients received either 6ml/kg or 12ml/kg mechanical ventilation. The initial findings of the ARDS Network were reported in year 2000 (2). The results have shown that ALI/ARDS-related death can be reduced with 22% in the lower tidal volume ventilation. Another beneficial outcome was the number of ventilator-free days in the lower tidal volume group. The ARDS Network group has continued to conduct studies related to ALI with more or less success (3-5). The database created by the "Network Group" is still target of intensive data mining (6, 7).

Recent research discoveries further added excitement to the field suggesting that mechanical ventilation itself can cause damage the lungs and induce systemic effects in the body (8).

The latest aspect of ALI-related medical care and research is the 2003 epidemic of severe acute respiratory syndrome (SARS) in South-Eastern Asia and Canada. The fatal acute lung injury caused by viral infection highlighted the importance of new therapeutic approaches in lung injury.

2.2 Definitions

There is confusion in the use lung injury-related terminology. To unify definitions international consensus conferences were held in 1994 and 1998 (9, 10). Since these conferences new ALI-related definitions have appeared in medical literature. I cited all common definitions related to the disease (Figure 1).

Acute lung injury

Acute lung injury is defined as a syndrome of acute and persistent lung inflammation with increased vascular permeability. Three clinical features characterize ALI:

1.Bilateral radiographic infiltrates.

2.A ratio of the partial pressure of arterial oxygen to the fraction of inspired oxygen (PaO2/FiO2) between 201 and 300 mmHg, regardless of the level of positive end-expiratory pressure (PEEP). The PaO2 is measured in mmHg and the FiO2 is expressed as a decimal between 0.21 and 1.00.

3.No clinical evidence for an elevated left atrial pressure. If measured, the pulmonary capillary wedge pressure is 18 mmHg or less (9).

Acute respiratory distress syndrome

The definition of acute respiratory distress syndrome (ARDS) is the same as acute lung injury except that the hypoxia is worse, requiring a PaO2/FiO2 ratio of 200 mmHg or less, regardless of the level of positive end expiratory pressure (PEEP). However, the distinction between acute lung injury and ARDS is somewhat arbitrary, since the degree of gas exchange disturbance does not correlate reliably with the extent of the underlying pathology. Furthermore, the severity of hypoxia does not impact predictably on clinical course or survival (10). ARDS typically develops over 4 to 48 hours and persists for days to weeks (11). Subacute or chronic lung diseases, such as sarcoidosis and idiopathic pulmonary fibrosis, are excluded from the definition of ARDS even though they may meet other elements of the definition.

"Mild acute lung injury"

A population having ALI-non ARDS with a PaO2/FiO2 ratio between 200 and 300 (12).

Ventilator-induced lung injury

Ventilator-induced lung injury (VILI) is defined as lung injury as acute lung injury directly induced by mechanical ventilation in animal models. Since VILI is

usually indistinguishable morphologically, physiologically, and radiologically from the diffuse alveolar damage of acute lung injury, it can only be discerned definitively in animal models (10).

Ventilator-associated lung injury

Ventilator-associated lung injury (VALI) is defined as lung injury that resembles ARDS and that occurs in patients receiving mechanical ventilation. VALI may be associated with preexisting lung pathology such as ARDS. However, unlike VILI, one cannot be sure that VALI is caused by mechanical ventilation; rather, VALI is associated with mechanical ventilation (10).

Barotrauma

Barotrauma is defined as extraalveolar air. Barotrauma occurs in patients with ARDS because the lung is severely injured and/or overdistened during mechanical ventilation. Overdistention may play a role in the pathogenesis of VALI and barotrauma was used as a marker of VALI or a synonym of VILI in the past. However recent research indicates that the mechanism of VALI is complex requires repeated mechanical insults, while barotrauma is limited to a single event of overinflation of the lungs. Therefore barotrauma is an insensitive and non-specific marker of VALI (10, 13).

Biotrauma

Biotrauma is new terminology in ALI. Scientists refer to biotrauma as a consequence of proinflammatory cytokine release from the lung caused by injurious mechanical ventilation. Biotrauma is the held responsible for multiple organ failure following VILI (8, 14).

Volutrauma

Volutrauma is lung injury caused by high-volume mechanical ventilation. High volumes lead to the overdistention of the alveoli. The morphology of damage is identical to that of barotraumas (15, 16).

Multiple organ failure, multiple organ dysfunction syndrome

Multiple organ failure (MOF) refers to the presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention. The multiple organ dysfunction syndrome (MODS) is classified as either primary or secondary. Primary MODS is the result of a well-defined insult in which organ dysfunction occurs early and can be directly attributable to the insult itself (e.g., renal failure due to rhabdomyolysis). Secondary MODS is organ failure not in direct response to the insult itself, but as a consequence of a host response (e.g., ARDS in patients with pancreatitis). In the context of the definitions of sepsis and systemic inflammatory response syndrome (SIRS) , MODS represents the more severe end of the spectrum of severity of illness characterized by SIRS/sepsis (17).

MODS is not directly related to ALI, but lung injury often leads to multiple organ dysfunction (15). Animal models suggest that in VILI proinflammatory cytokines released to the systemic circulation may cause MODS. Furthermore mechanical ventilation can cause the translocation of bacteria to the circulation leading to a septic condition (18, 19). These theories is debated at present (20). However I would like to include this idea in my thesis since it is considered as a keystone of new approaches to ALI by prominent investigators in this field (21, 22).

2.3 Epidemiology of acute lung injury/acute respiratory distress syndrome

In a recent analysis of the ARDS Network trial Gross and colleagues found that the incidence of ALI in the United States is 22.4 cases/ 100,000 population when crude numbers are measured (23). Previous studies show a similar range of numbers in a variety of countries (24-28). However Gross and his colleagues suggest that the real incidence is higher in the USA, is 62.4/100,000, because only hospitals with more than 20 intensive care unit beds reported ALI cases. This could be due to the fact that smaller hospitals reported only ARDS, the more severe form of ALI (23). The incidence (new cases/100,000 population) of ARDS varies between 3 and 88 according to international studies, depending whether patients with "mild ALI" are included in the analysis (23). In critically ill patients in intensive care units (ICUs) the prevalence of ALI/ARDS is 5-15% (28).Since the mortality rate of ARDS is very high most examined factors are associated with death. The origin of the underlying disease and the severity of the acute illness contributes to the outcome. Non-pulmonary organ failures, especially severe sepsis or shock have a major impact on the prognosis (29-31). The role of severity of ALI, especially the prognostic value of PaO2/FiO2 is debated. Two epidemiologic studies suggest that the outcome of ALI and ARDS is similar (27, 28). At this point it is unclear whether patients with "mild ALI" and with ARDS have different chances for survival(32-34).

2.4 Etiology of acute lung injury

Approximately 10 to 15 percent of patients admitted to an intensive care unit and up to 20 percent of mechanically ventilated patients meet criteria for ARDS. The mortality rate is appreciable and varies with cause, with most patients dying of

multiorgan system failure rather than isolated respiratory insufficiency. Current cigarette smoking predisposes to hospital presentations of ARDS. Since the lower airways are one of the most harmed organs of the body a large variety of harmful noxa and trauma lead to acute lung injury. The most common causes of ALI are listed in table 1. More than 60 causes of ARDS have been identified, however most cases are due to a few predisposing conditions. The major risk conditions for ARDS are sepsis (38-43%), documented aspiration of gastric content (30-37%), pneumonia (28-36 %) and chest contusion (11-17%) (12, 28, 35). Brun-Buisson and colleagues found that (based on 6522 ICU admissions in 78 hospitals in 10 European countries) in their population that mortality is the highest among patients where the underlining disease is sepsis (43%), lower in patients with pneumonia (36%) and the lowest is in association with trauma (11%). In Table 2, I listed their subgroup analysis of etiology of ALI/ARDS (12).

2.5 Pathology of acute lung injury

The pathological features of ALI are called diffuse alveolar damage (DAD). The pathogenesis of DAD can be divided into three phases:

1.exsudative phase of edema and hemorrhage

2. proliferative phase and

3. fibrotic phase.

The second 2 phases are often merged and mentioned as fibroproliferative phase.

1. Exudative phase

The exudative phase includes the first 4-7 days after the onset of injury. The lungs of patients, who die in this early phase of injury, appear to be hemorrhagic, the airducts are dilated, and the alveolar parenchyma is collapsed. The process is usually

diffuse, but can be regionally distributed. The earliest histological changes are capillary congestion, intestinal and alveolar edema and intra-alveolar hemorrhage. The most prominent pathologic feature of the exudative phase is the eosinophilic hyaline membranes in the terminal alveolar ducts. Hyaline membranes are composed of fibrin, surfactant and cellular debris. Alveolar epithelial cells adjacent to hyaline membranes damaged first especially along the tips of alveolar septa (Figure 2). The reason for early formation of hyaline membrane at this location is unknown. It is suggested that the relatively high concentration of oxygen and the drying effect of high inspiratory pressures during mechanical ventilation can play a role in the localization. Endotelial swelling, the denudation of capillary basement membranes and intravascular fibrin accumulation, marks endothelial injury. The presence of megakariocytes is linked to thrombus formation. Neutrophil leukoctyes recruitment is an important pathologic feature of ALI. Neutrophils are present in the alveolar septi and in the alveoli. The alveolar septum is largely widened by edema. This phenomenon can be way more severe than other morphological changes. Epithelial injury is usually more striking than endothelial. The rupture of the type 1 pneumocyte layer leaves a denuded basement membrane, which is adhered to hyaline-fibrin. Type 2 cells are more resistant and maintain capacity to differentiate to type 1 cells. Since the alveolar layer is ruptured fluid from the vessels can freely enter to the alveolar space causing edema (36).

2. Proliferative phase

The proliferative phase occurs between day 7 and 21 however in some cases type 2 cell proliferation is seen as early day 3. During this phase organization, inflammation and fibrosis becomes prominent in the lung parenchyma. When macroscopically observed the lung is red-gray and slippery by touch due to the

presence of newly formed connective tissue. Zones of completely destroyed airspaces alternate with distended air ducts. Microscopically, type 2-cell proliferation on the alveolar surface is the most important feature of this phase. These cells try to seal the injured alveolo-capillary unit. Proinflammatory cytokines, TNF-α, and IL-1β, are produced in these cells that may contribute to further progression of the injury (37, 38). Within the alveolar septa fibroblast and myofibroblasts proliferate and break the alveolar basement membrane, completely destroying the original alveolar structure. Fibroblasts convert the edema fluid to granulation tissue and collagen deposition takes place. Epithelial cells migrate over the surface of the granulation tissue also participate in transforming the intraalveolar exudates into connective tissue. This process occurs everywhere from the terminal bronchioli to the alveoli but most prominent in the alveolar duct (36). At the end of the proliferative phase the lung architecture is completely remodeled that is observed as thickened alveolar septi by light microscopy (Figure 3).

3.Fibrotic phase

By the 3-4 weeks the lung architecture can be completely remodeled, fibrocystic changes or traction bronchiectasis can be observed. Massive collagen deposition is present in the alveolar septi. Honeycombing is similar to the one seen in idiopathic pulmonary fibrosis but airspaces are usually smaller. These end-stage lungs are often observed in other chronic pulmonary conditions as well showing the lung's identical response to inflammation and/or injury.

The role of vessels in acute lung injury

Pulmonary vascular injury and pulmonary hypertension are frequent complications of ARDS. The severity of pulmonary vascular lesions correlate with the duration of the damage (36) (Table 3). In early ARDS vasoconstriction,

thromboembolism and interstitial edema contributes to increased pulmonary arterial pressure. During the fibrous process the connective tissue obliterates microvessels and increased arterial muscularization causes pulmonary hypertension. Microthrombi (<1mm in diameter) are usually present during the exudative phase while macrothrombi can be present in any phase of the disease.

2.6 Repair mechanisms in acute lung injury

Fibroproliferation

The cellular and molecular mechanism that determines whether a patient with ALI/ARDS will develop pulmonary fibrosis is not yet determined. Levels of procollagen III, a byproduct of collagen synthesis, are elevated in the BALF of patients with ARDS. Elevated procollagen levels were detected as early as on the 1st day of ALI. Pulmonary edema fluid taken from patients with ARDS is capable to activate fibroblasts. This process depends in part on bioactive IL-1 (39).

Apoptosis

Programmed cell death plays an important role in the resolution of inflammation and the repair process in ARDS. Type II hyperplasia and transformation to type I cells is mediated through apoptosis. The clearance of neutrophils from the airspace is also maintained by apoptosis and by phagocytosis of macrophages. Disturbed apoptosis of neutrophils is also important in ARDS (see in Discussion in *Specific inflammatory cells* section). An important apoptotic pathway mediator the soluble death receptor CD95 (Fas)-ligand has been shown to be elevated in the BALF of patients with ALI/ARDS (40). The concentration of soluble Fas-ligand and soluble Fas is increased in the edema fluid of ARDS patients. Immunostaining showed increased alveolar epithelial stainging with Fas ligand and Fas in patients who died

from ARDS(41). These findings indicate that the Fas system is activated in the lungs of patients with ALI/ARDS and may play an important role in the initiation of apoptosis.

2.7 The mechanism of ventilator-induced lung injury

Ventilator-induced lung injury is a major focus of ARDS research. Lung injury created by mechanical ventilation is identical to other types of acute lung injury (42). Simple cellular models provided us evidences that alveolar cells produce inflammatory cytokine when stretched (43, 44) (Figure 5). In VILI, lung overdistention by large volume or pressure mechanical ventilation leads to acute lung injury (13). Mechanical ventilation is often supplemented with other forms of injury such as LPS injection, cecal ligation or hypochloric acid instillation to complement the effect (two-hit models). In these complex animal models the disruption of alveolo-capillary barrier leads to pulmonary edema and diffuse inflammation. The potential mechanism of VILI is shown in figure 4. However recent and widely debated data shows that mechanical ventilation alone can lead to ARDS (single-hit models)(8, 21).The three major factors that lead to lung injury are: 1. inflammation, 2. barrier disruption and 3. decreased edema clearance.

1.Inflammation

Injurious mechanical ventilation (20ml/kg tidal volume or above) resulted in increased proinflammatory cytokine production in rodent models (TNF-α, MIP-2, IL-6, IL-1β) (18, 21, 45). It was first shown in an ex vivo lung model that higher tidal volume ventilation lead to higher cytokine levels than moderate volumes (30 ml/kg versus 12ml/kg)(8). Ranieri and colleagues in a human study confirmed these findings. Patients with ARDS, ventilated with high-tidal volume (mean 11.1ml/kg)

produced more TNF-α, MIP-2, and IL-6, IL-1β levels in their BALF than those ventilated with low tidal volume (mean 7.6ml/kg). High volume mechanical also induces neutrophil activation and infiltration to the alveoli (22) when compared to low tidal volume.

2. Alveolo-capillary barrier disruption

Disruption of the alveolar-capillary barrier is an important mechanism responsible for the formation of alveolar edema, which is a characteristic of VILI. This loss of compartmentalization combined with the ventilator-induced amplification of inflammation in acute lung injury may also be important mechanism of multisystem organ failure, one of the most common causes of death in ARDS (42).von Bethman and colleagues reported that proinflammatory cytokines TNF-α and IL-6 levels significantly increased in the perfusate buffer of isolated perfused and ventilated mouse lungs (21). The ARDS Network low tidal volume study also showed lower IL-6 levels in the BALF when the animals were ventilated wit reduced tidal volume (2). Several recent studies examined the effect of mechanical ventilation on the translocation of bacteria from the lung to the bloodstream. Animals ventilated with higher tidal volume develop bacteremia more frequently than those ventilated with protective ventilator strategies (46-48).

3. Alveolar edema

That alveolar edema is induced by mechanical ventilation was first shown by Webb and Tierney in a classical experiment where rats were ventilated with high volume (49). They have also discussed that the use of positive-end expiratory pressure (PEEP) can reduce edema formation. Edema fluids fill alveoli and promote airspace collapse by inactivating surfactant and filling airways. The loss of lung volume leads to heterogeneity in the lung resulting in even greater overdistention of the alveoli.

Therefore is the edema fluid clearance is decreased a vicious cycle of airspace edema leading to greater overinflation and shear stress will ensue. Lecuona and colleagues investigating edema clearance found that high-volume mechanical ventilation could reduce energy-dependent sodium transport mechanisms. This effect was circumvented with the instillation of β-adrenerg agonists (50). In clinical studies preserved fluid clearance correlates with improved survival (51).

2.8 Diagnostic methods in acute lung injury

The methodology applicable to clinical studies is more limited than what is applied in animals. The most commonly used techniques with their advantages and disadvantages are listed in Table 4. A wide spectrum of sampling methods helps us to confirm the nature of the disease and follow its course.

2.9 New therapeutic approaches in acute lung injury

Acute lung injury is a commonly observed in its advanced form ARDS in medical intensive care units (MICU). Since ALI has multiple etiological characters there is no standard therapy for ARDS. However mechanical ventilation support is a key element of ARDS therapy regardless of etiology. Mechanical ventilation has gone through an enormous development since it was first introduced in the 1960`s. Despite new technical advances until recently the mortality of ARDS has remained high, close to 50%. Furthermore, high tidal volume (volutrauma) or high-pressure (barotrauma) mechanical ventilation can contribute to acute lung injury in the form of ventilator-associated lung injury (VALI) The first big achievement in reducing mortality of ARDS was the published results of ARDS Network study group in year 2000. A 22% decrease in mortality was observed when patients were ventilated with lower tidal

volume (6ml/kg) and compared with patients ventilated with conventional 12ml/kg tidal volume (2). These results show that mechanical overstretch of alveoli further propagates alveolar injury in the inflamed lower airways. Another interesting consequence of this study is the fact ideal alveolar oxygenation is not the most important issue during mechanical ventilation in ARDS. Today it is recommended to use low pressure-low tidal volume ventilation in ARDS with a plateau pressure less than 30cmH_2O or 6ml/kg tidal volume. Relative hypercapnia (permissive hypercapnia) is well tolerated in these patients. Amato and colleagues found that positive end expiratory pressure (PEEP) ventilation and frequent recruitment maneuvers has beneficial effects on arterial oxygenation allowing the reduction of inspired oxygen fraction (FiO_2) during mechanical ventilation (52). In early ARDS, when tracheal intubation is not necessary noninvasive ventilation is recommended. Partial liquid ventilation, high frequency mechanical ventilation and prone positioning was also tired in ARDS but the results did not show the benefits of these alternative ventilatory techniques in ARDS (53).

Recently inhibitors of proinflammatory cytokine TNF-α and IL-1β were given to patients with ARDS, but no benefits were documented (54).

2.10 A novel approach to lung injury; the use of low dose inhaled carbon monoxide as an antiinflammatory agent

It is established that mechanical ventilators that apply high volumes and pressures can lead to increased alveolar-capillary permeability (13). This loss of compartmentalization results in increased fluid influx to the alveoli from the capillaries, causing pulmonary edema. The injured or ruptured cells attract neutrophil leukocytes and activate alveolar macrophages, causing inflammation in the lung (13).

Tremblay and colleagues have suggested that ventilation can provoke an inflammatory response in the distal airway and alveolar cells(8), manifested by increased production of pro-inflammatory cytokines. It is speculated that the released proinflammatory cytokines could enter the circulation causing inflammation in other systemic organs. In addition, the previously diseased or injured lungs are more susceptible to subsequent mechanical ventilation, releasing more pro-inflammatory cytokines than healthy lungs, perhaps reflecting the cumulative effects of multiple injuries (42).Accumulating data exist in the literature supporting the paradigm that the stress inducible heme oxygenase-1 (HO-1) or its catalytic by-product, carbon monoxide (CO), can confer potent cytoprotective effects in various models of tissue and cellular injury (55-61). One mechanism by which HO-1 or CO mediate a cytoprotective effect is via its potent antiinflammatory properties (55, 60, 62). Our laboratory has recently demonstrated that exogenous administration of low concentration of inhaled CO can markedly decrease lung inflammation and confer potent cytoprotection in various tissue injury models(55-61).

2.11 Specific aims

Aim #1: Gene expression analysis of mouse lung genes following acute lung injury.

1. To determine which genes show significant expression changes in acute lung injury. Functional grouping of these genes.
2. To describe groups of genes modified by LPS-induced lung injury and/or high-volume mechanical ventilation. Description of a distinct group of genes modulated only by mechanical ventilation injury.

Aim #2. To describe novel target genes in ventilator-induced lung injury.

1. To confirm the gene expression changes of target genes on the RNA and protein level.
2. The role of amphiregulin, a new candidate gene in acute lung injury

Aim #3: To determine the effects mechanical ventilation in acute lung injury

1. To demonstrate the effects of mechanical ventilation on alveolar edema and inflammation in LPS-induced lung injury
2. To describe heme oxygenase activation and induced cytokine production in ventilator-induced lung injury (TNF-α, IL-10)

Aim #4: To describe and confirm the aniinflammatory effects of low dose, inhaled CO in ventilator-induced lung injury

1. To present the effects of CO on the direct and indirect indices of acute lung injury (altered inflammatory cell infiltration, reduced proinflammatory cytokine levels).
2. To describe the effect of CO cardio-respiratory function (blood pressure, blood gases)
3. To determine the mechanism through witch intracellular pathway CO provides anti-inflammatory effects in ventilator-induced lung injury. CO exerts antiinflamatory effects independent of NF-κB and AP-1 involving p38 mitogen-activated protein kinase.

3. Materials and methods

In my series of animal experiments I was interested in mechanism of VILI/ARDS. This includes gene expression changes, intracellular signaling pathways activation, cellular involvement, pulmonary and circulatory effects and animal behavior due to mechanical ventilation. Furthermore, I experimented with a new therapeutic solution; the application low-dose inhaled carbon monoxide (CO) to

reduce lung injury in mechanically ventilated rodents. I would like to introduce my research findings through 2 animal models I developed and worked with at the University of Pittsburgh, Pittsburgh, PA, USA and at the Research Center in Borstel, Germany.

3.1 Animal models

1. To assess the gene expression changes during mechanical ventilation I used an isolated, perfused and ventilated mouse lung model developed by Professor Stefan Uhlig in Germany. This model is often called *ex vivo* to represent its transitional position between *in vitro and in vivo* experimental settings. The *ex vivo* model has the advantage of keeping the chest almost intact without loosing the chest wall component of breathing and at the same time applied high pressure mechanical ventilation does not compromise cardiac function as often seen in models of VILI (42). The detailed picture of the model is shown in Figure 6.

In brief, 9 Balb/c mice (22-30g) were used. The animals were anesthetized with 160mg/kg pentobarbital sodium (Nembutal; Wirtschaftsgenossenschaft Deutscher Tierärzte, Hannover, Germany). Following tracheostomy was performed and a canula was inserted in the treachea. We ventilated the animals through the canula with 90 breaths/minute with 200µl tidal volume of room air using a rotary vane compressor pump (VCM; Hugo Sachs Electronic, March-Hugstetten, Germany). Subsequently, laparotomy was performed and the diaphragm was removed. The mice were heparinized, exsanguinated and the abdomen was removed. A ligature was put around the pulmonary artery and the aorta. Canulas were inserted in the left atrium and the pulmonary artery and the vessels were constantly perfused with RPMI 1640 tissue culture media supplemented with 4% bovine serum albumin (Biochrom, Berlin,

Germany). The flow rate of the perfusate was 1 ml/minute generated with a peristaltic pump (Ismatec MS Reglo). Following the thorax was removed and a chamber was placed over the animal. Once the chamber lid was closed we ventilated the mice with negative pressure for 1 hour with an end-expiratory pressure of -3 cm H_2O and an end-inspiratory pressure (EIP) of -10 cm H_2O. Every 5 minutes the lungs were recruited automatically with –20 cmH_2O (TCM; Hugo Sachs Elektronik). Subsequently, they were randomized in 3 groups and ventilated for the next 3 hours (n=3/group). Control: ventilation with -10 cm H_2O EIP, LPS: ventilation with -10 cm H_2O EIP in the presence of 3mg/kg Escherichia coli bacterium lipopolysaccharide (LPS), Serotype O127: BO (Sigma, St. Louis, MO) in the perfusion media, (3) overventilation with -25cm H_2O EIP.

2. In a second, in vivo model of lung injury I accessed the cellular changes, pulmonary edema formation, cytokine production and intracellular signaling pathway activation following high volume (26ml/kg) mechanical ventilation without PEEP (63).Furthermore I tested the anti-inflammatory effect of low-dose inhaled CO in the model. Adult male Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN), weighing 275-375g (n=88). Rats were allowed to acclimate for 1 week with rodent chow and water ad libitum. The rats were randomized to either receive 3mg/kg Escherichia coli bacterium lipopolysaccharide (LPS), Serotype O127: BO (Sigma Chemical Co., St. Louis, MO) in 0.25ml Phosphate Buffered Saline (PBS) or PBS alone, injected into the tail vein under ketamine (75mg/kg ip.) and acepromazine (2.5mg/kg ip.) anesthesia (Sigma Chemical Co., St. Louis, MO). After one hour of spontaneous breathing, tracheotomy was performed on the rats and a 14-gauge canula was inserted into the trachea. We designed 7 experimental conditions: control, LPS,

ventilation, ventilation/CO, LPS/ventilation, LPS/ventilation/CO and SB203580/LPS/ventilation/CO (Figure 7). Pairs of CO treated and non-treated animals were formed and treated one after another. PBS treated animals were randomly chosen to conditions control, ventilation and ventilation/CO LPS treated animals were randomly chosen to conditions LPS, LPS/ventilation and LPS/ventilation/CO. Animals in condition SB203580/LPS/ventilation/CO were littermates of rats treated according to other conditions. These animals were randomized prior the beginning of the experiments. After tracheostomy, animals treated according to condition ventilation, ventilation/CO, LPS/ventilation, LPS/ventilation/CO and SB203580/LPS/ventilation/CO were connected to volume cycled rodent ventilator (Harvard Rodent Ventilator, Model 680, Harvard Apparatus, South Natic, MA). They were ventilated with 26 ml/kg tidal volume, at 38/min respiratory rate without PEEP. The ventilator delivered room air or 10-250 parts per million CO mixed with room air to the animals. The ventilation period was 15 minutes to 4 hours.

Muscle relaxation was performed by the intraperitoneal injection of pancuronium bromide (1mg/kg/h) (Sigma Chemical Co., St. Louis, MO). Administering stepwise injections and withdrawals of 0.5-1cm^3 of air to the transpulmonary pressure of 25cm H2O recruitment was carried out every 30 minutes (HP45 pressure transducer, Validyne Engineering Co., Northridge, CA). Additional anesthesia was induced with ip. injection of ketamine (75mg/kg) and acepromazine (2.5mg/kg) if needed. The animals were sacrificed with an overdose of ketamine (150mg/kg) at the end of the experiment.

Assessment of lung injury

To determine the magnitudes of lung injury caused by LPS, ventilation, and LPS/ventilation in I performed two-hour experiments. LPS animals were sacrificed 2 hours after injection of LPS. In the LPS/ventilation condition, animals received LPS injection, and allowed to spontaneously breath for 1hour, and then treated with 1hour mechanical ventilation. Ventilation only animals received 2 hours of mechanical ventilation, and then sacrificed.

SB203580 p38-kinase inhibitor experiment

SB203580/LPS/ventilation/CO animals were injected intraperitoneally with 20mg/kg SB203580 p38-kinase inhibitor (Calbiochem, La Jolla, CA) 30 minutes prior to the experiment (n=6). We used water-soluble, SB203580-hydrochloride dissolved in 0.6ml PBS per animal. Following the animals received LPS injection, and allowed to spontaneously breath for 1hour, and then treated with 1hour mechanical ventilation. The control animals were treated according to LPS/ventilation/CO condition. The ventilation time was 1 hour. The control animals received in addition, 0.6ml ip. PBS injection (n= 3). Since PBS did not have an effect on the IL-10 levels, later it was combined with other LPS/ventilation/CO data. Three lungs were used for histology.

Time course experiment for TNF-α BALF level

Animals treated as LPS/ventilation condition were ventilated for 15 minutes to 3 hours to describe the effect of the treatment on TNF-α BALF levels (n=3/timepoint).

Four-hour ventilation experiment

To examine whether CO inhalation can effect neutrophil leukocyte infiltration to the alveoli we performed LPS/ventilation and LPS/ventilation/CO condition experiments where animals received LPS injection, and allowed to

spontaneously breath for 1hour, and then treated with 4-hour mechanical ventilation. N=3 animals per condition (Table 9).

Arterial blood pressure and blood gas measurements

A 24-gauge angio catheter was inserted into the right carotid artery of LPS/ventilation and LPS/ventilation/CO treated animals (n=3/condition). Blood was sampled for blood gas analysis and we measured mean arterial blood pressure (Pā) via a blood pressure transducer (UFI, Model 1050-1, Morro Bay, CA) zeroed at the midthorax. The blood gases were measured in the beginning and at the end of the experiment with a Radiometer ABL5 (Radiometer, Copenhagen, Denmark) blood gas analyzer. Blood pressure was continuously measured throughout the experiment (Table 5).

CO dose-response experiment and CO exposures

Rats treated according to LPS/ventilation/CO condition were exposed to room air (0 ppm) or different concentrations of CO (10, 100, 250 parts per million) for 1 hour through the rodent ventilator (n=6 animals for 0 and 250ppm, n=3 for 10 and 100ppm of CO). Animals treated as LPS/ventilation condition served as 0 ppm. In case of all other experiments, we treated the animals with 250ppm CO. For CO exposures CO was mixed with room air. Valley National Gases (Wheeling, WV) provided the CO gas chambers. The concentration of CO was adjusted via a flow meter; the mixed gas runs to a chamber, which is connected to the rodent ventilator. The applied gas pressures were always the same. A CO analyzer was used to measure CO levels (Interscan, Chatsworth, CA) in the chamber. Gas samples were introduced to the analyzer through a port in the top of the chambers at a rate of 1l/min and were analyzed by electrochemical detection, with a sensitivity of 10-600

parts per million. After approximately 5 minutes, equilibrium was reached in the CO concentration. Concentration levels were measured hourly.

3.2 Gene expression analysis

1. Microarray

To analyze gene expression changes in the mouse model of VILI, I used microarray technology. Microarray technology allows to study gene expression of a significant fraction of the genome and to identify candidate genes critical to a variety of lung diseases including chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, lung cancer and pulmonary hypertension (64-68).

Total RNA was extracted from lung tissue with Trizol (Invitrogen, Carlsbad, CA). Labeled complementary RNA was generated and hybridized to Codelink Uniset I Bioarrays as recommended by the manufacturer (Amersham Biosciences, Piscataway, NJ). The schematic description of microarray technique is shown in Figure 8. In brief, we synthesized double-strand cDNA using reverse transcriptase, dNTP and DNA polymerase from 0.2-2 μg total RNA/animal. After purifying the cDNA in vitro transcription was performed in the presence of biotin labeled 11-UTP to produce cRNA (CodeLink Expression Assay Reagent Kit, Amersham). The quality of the cRNA was accessed with denaturing gel electrophoresis. Fragmented cRNA from each animal was hybridized individual genearray slides containing the oligo sequences of 10500 genes (Uniset Mouse 1 Array, Amersham). The slides were incubated at the same time, under the same conditions to enable the comparison among the different samples. We used Alexa-Streptavidin dye conjugate for detection. The bioarrays were scanned and analyzed (Codelink Expression Analysis scanner and software, Amersham). The expression data is generated by the comparison of light

emission of the background, positive control genes and the genes we desired to test. All arrays were average scaled to the same value. For analysis we filtered out genes that did not pass manufacturer recommendation for quality control. A total of 5612 valid genes were obtained for analysis. Statistical analysis was performed by Significance Analysis of Microarrays (SAM, Stanford, CA) program and Scoregene software package (Scoregene Package, available at http://compbio.cs.huji.ac.il/scoregenes/). In SAM, 200 permutations of the data were generated and significant differences between treatment groups were determined with t-tests (p-value<0.05). The False Discovery Rate (FDR, q-value<0.05) method was applied to correct for multiple testing. Additionally we used the non-parametric Threshold Number of Misclassification score (TNoM). Genes with TNoM=0 were considered significantly different. There was a 90% overlap between the genes identified with the two programs. A total of 567 significant genes were found.

2.Quantitative Real-Time (TaqMan) Polymerase Chain Reaction

Quantitative Real-Time (TaqMan) Polymerase Chain Reaction (QRT-PCR) was used to confirm microarray findings. I performed QRT-PCR for 5 significantly increased expression genes with overventilation and LPS treatment in the mouse model of VILI. TacMan PCR was performed as described previously(69). In brief, RT-PCR detects the accumulation of amplicon during the reaction. Previous methods (end-point PCR) were measuring RCR in the plateau phase where detection is less reliable. In TaqMan PCR, a 5`Nucleotide assay is used to detect PCR products the exponential phase of amplification with high precision. The PCR mastermix contains AmpliTaq Gold® DNA polymerase and an oligonucleotide called a TaqMan® Probe. The DNA polymerase has polymerase and 5` exo-nuclease activity (Figure 9A). The polymerase adds bases to the growing chain of DNA and subsequently it removes

DNA downstream impending its` capacity to synthesize the new standard. Taqman® Probe is designed to anneal to a specific sequence of template between the forward and reverse primers. The probe sits in the path of the enzyme as it starts to copy DNA or cDNA (Figure 9B). When the enzyme reaches the annealed probe the 5`exo-nuclease activity of the enzyme cleaves the probe (Figure 9C). The probe is designed with a high-energy dye termed a reporter at the 5` end, and a low energy molecule the quencher at the 3` end. When the probe is intact and excited by light, the reporter dye`s emission is suppressed by the quencher dye as a result of the close proximity of the dyes (Fluorescent Resonance Energy Transfer). When the probe is cleaved by the 5` nuclease activity of the enzyme the distance between the reporter and quencher dyes increases causing transfer of energy to stop. The fluorescent emission of the reporter increases and the quencher decreases. The increase of reporter signal is captured. The amount of signal increase is proportional to the amount of product being produced for a given sample. Commercially available Assay-on-Demand primer probe sets (Applied Biosystems, Foster City, CA) were used for IL-11: Mm00434162_m1, AREG: Mm00437583_m1, AKAP12: Mm00513511_m1, NUR77: Mm00439358_m1, and CYR61: Mm004487498_m1. PCR amplification of the cDNA derived from lung tissue (n=3) was performed in triplicate. Gene expression was measured relative to an endogenous reference gene, mouse β-glucuronidase (β-GUS). The results were $\log_2$ base transformed and the arithmetical means of 3 measurements were compared. Results are presented as mean± SD. Kruskal-Wallis test was performed for multiple group comparison and inter-group differences were analyzed with Wilcoxon Rank Sum Test with SPSS statistics software (SPSS, Chicago, IL). The significance level was set at $p<0.05$.

3.3 Cellular assays for the verification of gene expression results

Further verification of amphiregulin gene expression was assessed with protein expression analysis.

1.Tissue immunohistochemical-staining

Lung tissues were fixed with 2% para-formaldehyde, merged in 30% sucrose overnight and snap frozen. Sections of lung were prepared and immunostaining was performed as described (67) Amphiregulin goat polyclonal IgG primary antibody was used (Santa Cruz Biotechnology, Santa Cruz, CA). A representative picture per group was taken with an Olympus BX51 Fluorescent microscope (Olympus America, Melville, NY).

2.Western blotting

Protein was extracted from whole lung tissue as described previously (70). The total protein concentration was determined with a Coomassie Plus 200 Protein Assay (Pierce, Rockford, IL). Western blot analysis was carried out as previously described for amphiregulin (71). In brief: We performed Western blot analysis by immunoblotting 25μg of total protein from whole lung homogenate preparations. An equal amount 2x SDS sample buffer (0.125M Tris HCl (ph 7.40), 4%SDS, and 20%Glycerol) was added and the samples were boiled for 5 min. The samples were subjected to electrophoresis on a 4-12% SDS-poliacrylamide gel (Bio-Rad Laboratories) for 2 h at 20mA. The proteins were then transferred electrophoretically (Bio-Rad Laboratories) onto a polyvinylidene fluoride membrane (Immobilon, Bedford, MA) and were incubated 2h in Tris-buffered saline (TBS) and 1% Tween 20 (TBS-T) containing 5%nonfat powered milk. The membranes were than incubated for 2 hours with goat polyclonal primary antibody against amphieregulin (1:1000

dilution) (Stressgen Biotechnologies Corp., Victoria, BC, Canada, Catalogue No. OSA111). After 3 washes in TBS-T for 5 min. each, the membranes were incubated with goat anti-mouse immunglobulin G antibody (Cell signaling Technologies, Beverly, MA) for 2 hours. The membranes were then washed 3 times with TBS-T for 5 min each, followed by the detection of the signals with an enhanced chemiluminescence detection kit (Pierce, Rockford, IL). The p38 kinase activity assay was performed same lack using a kit, purchased from Cell signaling Technologies, Beverly, MA.

3.4Necropsy protocol, tissue and BALF analysis in the *in vivo* model of ventilator-induced lung injury

At the end of the experiment the abdomen and the chest of the animals was opened up. The left lung was isolated with surgical silk tight around the left main bronchus in 3 animals per every condition. The right lungs were inflated with 4ml of 2% paraformaldehyde to transpulmonary pressure through the tracheostomy canula for 1 minute and than harvested. Following, the lungs were fixed for 2 hours in 2% paraformaldehyde and submerged in 30% sucrose overnight. Next morning the tissue was snap frozen in liquid nitrogen and stored at –80C. The frozen tissues were embedded and stained with hematoxylin-eozin for histology. An experienced pathologist analyzed the lungs in a double-blinded fashion. The left lungs were harvested, snap-frozen and stored at –80 C for tissue protein, nuclear protein and RNA extraction. All other animals' lungs were lavaged twice using 8ml PBS solution. The lavage volumes were inserted and withdrawn 3 times. We gained 12-13ml BALF per animal. 100µl of BALF was mounted on glass slides by cytospinning for 10 minutes at 1000rpm (Cytospin 3, Shandon Scientific, Runcorn, England) and

stained with hematoxilin-eosin for differential cell count. Two hundred cells were counted per sample. The rest of the fluid was immediately centrifuged (2500rpm for 10 minutes) at +4C. The supernatant was stored at –20C for cytokine analysis. The pellet was resuspended in 1 ml PBS for quantitative cell count. The cells were counted in a hemocytometer (Hausser Scientific, Horsham, PA).

3.5 Cellular assays (ELISA, total protein measurement, western and northern blots) in the *in vivo* model ventilator-induced lung injury

1.ELISA

The cytokine analysis of BALF was carried out as described, using rat specific kits (R&D, Minneapolis, MN) (8). The lower detection limits of the kits are 4pg/ml and 13pg/ml respectively. The absorbance was detected at 450nm with plate reader (Versamax Microplate Reader, Molecular Devices, Sunnyvale , CA) .

2.Total protein measurement in bronchoalveolar lavage fluid

Total protein concentration of lung lavage fluid was determined by Coomassie Plus 200 Protein Assay (Pierce, Rockford,IL). Bovine serum albumine was used to construct the standard curve. The Coomassie Protein Assay was read by a Backman Coulter DU640 spectrophotometer (Backman Coulter Inc., Sommerset, NJ) at 595nm.

3.Western blot analysis

Western blot analysis was carried out as previously described for HO-1 and p38 MAPK phosphorylation (see above in *Cellular assays for the verification of gene expression results* and Reference#(60) .

4. Northern blot analysis

Total cellular RNA was extracted from lung tissue and Northern blot analysis was performed for HO-1 gene expression as previously described (59). Total cellular RNA was extracted from frozen lung tissue using Trizol (Invitrogen, Corp. Carlsbad, CA). Northern blot analysis were performed as previously described. In brief, 10μg of total RNA were electrophoresedon 1%agarose gel and then transferred to Zeta-Probe Blottting Membrane (Bio-Rad Laboratories, Hercules, CA) by capillary action and cross-linked with a UV Stratalinker (Stratagene, La Jolla, CA). EagleEye UV transluminator (Stratagene) was used to check the integrity of RNA. The membranes were then prehybridized in hybridization buffer (1%BSA, 7%SDS, 0.5M phosphate buffer,and 1mM EDTA) at 65C for 2 hours followed by incubation in hybridization buffer containing ^{32}P-labeled rat HO-1 cDNA at 65C for 24h. The membranes were then washed in wash buffer A (0.5%BSA, 5%SDS,40mM phosphate buffer, pH 7.0 and 1.0mM EDTA) for 15 min three times at 55C. Autoradiogram signals were detected on Kodak Biomax MR Film (Eastman Kodak Co. Rochester,NY).

3.6 Electrophoretic mobility shift assay (EMSA)

Mobility shift assays were performed as described by Barberis (59) with minor modifications.

1. cDNA and oligonucleotide probes

A full length of rat HO-1 c DNA was subcloned into pBlue-script vector, and *Hin*d III-*Eco*R I digestion was performed to isolate a 0.9-kb HO-1 cDNA insert. A 24-bp oligonucleotide (5`-ACGGTATCTGATCGTCTTCGAACC-3`) complementary to 18S rRNA was syntetized with a DNA synthetizer (Applied Biosystems, foster City, CA). HO-1 cDNA was labeled with [α-^{32}P]CTP with a random- primer kit (Boehringer Mannheim, Mannheim, Germany). The 18S rRNA oligonucleotide was

labeled with [α-^{32}P]ATP at the 3`-end with terminal deoxynucleotidyltransferase (Bethesda Research Laboratories, Gaithersburg, MD).

2. Electrophoretic mobility shift assay (EMSA)

The binding activity was determined after incubation of 4μg of nuclear protein extract with 10fmol (25,000-60,000 counts/min) of either a ^{32}P-labled 22-mer oligonucleotide encompassing the activator protein (AP)-1 site (5`-CTAGTGATGAGTCAGCCGCATC-3`,Stratagene) or a ^{32}P-labled 22-mer oligonucleotide encopassing Nuclear Factor (NF)κB site (5`-AGTTGAGGGGACTTTCCCAGGC-3`, Promega) in reaction buffer containing 10mM HEPES (pH 7.9), 1mM DTT, 1 mM EDTA, 80mM potassium chloride, 1μg of poly(dI-dC) and 4%Ficoll. After a 20-minutes incubation, the reaction mixture was electrophoresed on a 4% polyacrylamide gel. A gel was transferred to DE81 ion-exchange chromatography paper (Whatman, Maidstone, UK) and dried down before exposuture to autoradiographic film.

4.Results

4.1 Specific Aim #1.Gene expression profiling in a mouse model of isolated perfused and ventilated lung

We compared the gene expression patterns of -10 cm H_2O EIP ventilated (controls), -25 cm H_2O EIP ventilated (overventilation) and LPS-treated animals by microarray analysis. Figure 10 demonstrates that the lungs of the animals in the three treatment protocols exhibited distinct gene expression profiles. We identified 3 clusters of genes that behaved different in the 3 treatment groups. The expression of cluster A genes was decreased in the overventilated animals (OV) and unchanged in the LPS-treatment group (LPS). These genes show a generally lower expression.

Cluster B genes displayed decreased expression in the OV group but their expression was increased in the LPS group. Cluster C genes were increased by OV treatment. Many of them were also increased in the LPS group but to a lesser extent. Using two different statistical programs (Scoregene TNoM=0, SAM p-value<0.05) we identified 567 genes that had significantly different expression levels following LPS and/or OV treatment when compared to controls. A total of 182 genes were changed by OV and not by LPS. The expression of 36 genes was induced, and that of 146 genes decreased. Table 6 contains the list of well-known genes modulated only by OV. Included were genes of general metabolism, growth factor related genes, intracellular transport, one cytokine ligand and one antioxidant. The genes with decreased expression featured growth factor-related genes, cell cycle regulators, antioxidants, oncogenes, complement and coagulation related genes. Both LPS and OV regulated 385 genes. Among these the expression of 246 was increased and that of 139 decreased. Well-known genes that were affected by both LPS and OV are listed in Table 7, among those many are cytokines, transcription factors and apoptosis-related genes. Certain growth factor genes also appear, such as granulocyte-macrophage colony stimulating factor (GM-CSF) as well as Nur77. LPS and OV reduced the expression of certain well-known protein kinases, cell cycle regulators, transcription factors and growth factors.

Functional grouping of genes following lung injury

We assessed cellular functions that were modified by LPS treatment and/or overventilation. First we identified genes with increased or decreased expression by creating $\log_2$ base ratios of the gene expression results (lps/c, ov/c and ov/lps respectively). Following we used GeneXpress (http//: www.genexpress.stanford.edu)

to determine whether a specific cellular function annotations were enriched in one or more of the pairwise combinations (72). Only statistically significant enriched annotations are shown in Figure 11. Statistical significance was determined using hypergeometric model and corrected for multiple testing using FDR methods (67). A 95 % confidence interval was set for the FDR correction to correct for multiple testing. Findings were visualized on a color map where orange represents the up-regulation of gene expression related to the cellular function and blue stands for reduced expression in groups of genes related to the same function (Figure 11). Genes induced by OV but not by LPS were enriched for genes involved in cytoskeleton and cell communication. Genes induced only by LPS were enriched for genes involved in cytokine activity and in nucleic acid binding. Genes induced by both OV and LPS were involved in the immune response, carrier activity, and protein binding physiological processes (Figure 11). The list of cellular functions with the corresponding p-values and the number of statistically significant genes per functional annotation found among the 5612 valid genes is included in Table 8.

4.2 Specific aim #2.Genes with significant changes in expression

Based on gene clustering and gene scoring results we decided to validate the RNA expression of 5 target genes: Areg (Amphiregulin), Akap12 (A kinase anchoring protein), Cyr61 (Cystein-rich protein 61), Nur77 (nuclear receptor subfamily 4, group A, member 1) and Il11 (Interleukin-11). These genes are all found in cluster C (Figure 12) with significantly increased expression (Scoregene TNoM=0, SAM p-value<0.05) following overventilation and LPS-treatment (Figure 11). Table 3 contains the gene expression fold changes in the LPS and OV treatment groups when compared to controls. The increased mRNA expression of the 5 genes was validated by RT-PCR

(Figure13). Our findings were further substantiated by immunoblotting for amphiregulin. Amphiregulin protein expression was increased in the OV group when compared to the control and LPS treatment groups (Figure 14). Finally, we used the same antibodies to immunostain the lungs of the 3 experimental groups (Figure 15). Lungs subjected to overventilation showed increased amphiregulin staining compared to controls and LPS-treated lungs with primarily epithelial localization.

Specific aim #3.Ventilation enhances LPS-induced lung injury in rats

To determine the magnitudes of lung injury caused by LPS, ventilation, and LPS/ventilation we performed two-hour experiments. LPS animals were sacrificed 2 hours after injection of LPS. In the LPS/ventilation condition, animals received LPS injection, and allowed to spontaneously breath for 1hour, and then treated with 1hour mechanical ventilation. Ventilation only animals received 2 hours of mechanical ventilation, and then sacrificed. For all animals, we measured total cell number and total protein from the BALF. LPS or ventilation alone significantly augmented the total cell number measured in the lavage fluid, as did LPS followed by ventilation (Figure 16A). LPS treatment followed by ventilation also significantly enhanced the total protein levels in BALF whereas ventilation or LPS alone did not increase BALF protein levels (Figure 16B). Figure 17 demonstrates the hematoxylin-eosin stained histology of the lung tissue following 2 hours treatments. When compared to PBS treatment (Figure 17A), LPS or ventilation alone caused inflammatory cells infiltration into the alveolar septi and thickening of the alveolar wall (Figure 17B,C). The combined effect of LPS and ventilation was the most injurious that resulted in the destruction of the alveolar structure (Figure 17D). Greater magnification shows

infiltrating mononuclear leucocytes into the alveoli (Figure 17E). The histology further supports the finding that ventilation further enhances LPS-induced lung injury.

Pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) levels dramatically increased in BALF after LPS/ventilation treatment compared with the control or ventilation alone treatment conditions (Figure 18A). Kinetic experiments show significantly elevated TNF-α levels in LPS/ventilation treated animals after 30 minutes ventilation. The maximal TNF-α level was measured after one-hour ventilation on the 3-hour ventilation time course (Figure 18B).

VILI induces HO-1 expression

In order to examine whether VILI can induce expression of the stress inducible HO-1, we performed Northern and Western blot analyses to determine HO-1 expression levels in the lung tissues after ventilation. As depicted in Figure 19, LPS or mechanical ventilation alone increased HO-1 gene and protein expression. The use of LPS, as a primer of lung injury, followed by ventilation increased HO-1 mRNA and protein expression the most when compared to controls (Figure 19A, B). These data suggest that HO-1, an important cellular stress response gene product, may play a role in defense against VILI.

4.4 Specific aim #4. Inhaled CO inhalation attenuates ventilator-induced lung injury

Inhaled CO significantly reduced the total cell number increased by LPS/ventilation in the BALF (Figure 20A). Ventilation alone with CO (ventilation/CO condition) markedly reduced the total cell number, but it did not reach significance. CO did not affect cell count in control and LPS treatment

conditions (data is not shown). CO treatment did not affect the elevated total protein levels (Figure 20B).

We observed a dose-dependent decrease of the pro-inflammatory cytokine TNF-α in BALF when the animals inhaled CO during mechanical ventilation (Figure 20C). We also measured the anti-inflammatory cytokine interleukin-10 (IL-10) in the BALF. LPS, ventilation or their combination did not affect IL-10 levels. CO treatment in LPS/ventilation/CO condition significantly increased IL-10 in the BALF. The effect was not observed in ventilation/CO condition (Figure 20D). CO did not have an effect on cytokine levels in control and LPS treatment conditions (data not shown). Modest decrease in hypercellularity and inflammation was observed in tissue histology after CO treatment (Figure 17F).

Differential cell count showed significantly reduced number of macrophages in the BALF after 2 hours treatment with 250ppm CO mixed with room air (Table 9). The number of neutrophil leukocytes in the BALF at this timepoint was negligible. To examine whether CO inhalation can effect neutrophil leukocyte infiltration to the alveoli, we performed LPS/ventilation and LPS/ventilation/CO condition experiments where animals received LPS injection, and allowed to spontaneously breath for 1hour, and then they were mechanically ventilated for 4 hours. Treatment with 250 ppm CO resulted in significantly reduced total cell count and neutrophil cell count in the BALF (Table 9). This finding suggests that CO may also reduce lung injury via inhibiting neutrophil leukocyte infiltration into the alveolar space.

Inhaled CO exerts negligible effects on hemodynamics and oxygenation

In order to confirm that mechanical ventilation and low dose inhaled CO used for our studies did not exert untoward effects on hemodynamics and gas exchange; we

measured blood pressure and blood gases in LPS/ventilation and LPS/ventilation/CO conditions. Following tracheostomy a canula was inserted in the right carotid artery and blood pressure was continuously measured during the ventilation. Blood was sampled from the canula for blood gases in the beginning and the end of mechanical ventilation. We did not observe statistically significant differences in blood pressure, pH, PCO_2, and PO_2 in LPS/ventilation and LPS/ventilation/CO conditions during the course of the experiment. Inter-group differences were not significant either. LPS treatment or ventilation alone had no effect on the hemodynamic of the animal model (data not shown). The carboxy-hemoglobin level was significantly elevated in CO treated animals, as expected (Table 2 and 3).

Mechanisms of CO-induced anti-inflammatory effects

Transcription factors are involved in cellular stress-induced gene expression and regulate biological processes including inflammation, cell proliferation and cell survival, all of which are important in conferring protection against cell and tissue injury (10). We examined whether transcription factors such as AP-1 and NF-κB are involved in CO-induced anti-inflammatory effects in VILI. LPS and ventilation both induced activation of AP-1 and NFκB (Figure 21A and B); however, CO treatment did not modulate the activation of AP-1 or NFκB binding activity compared with ventilation or LPS/ventilation treatment.

Another intracellular pathway that is activated in lung injury is p38 mitogen-activated protein kinase (MAPK) (23). In our model LPS and ventilation increased activation of p38 MAPK; CO significantly increased p38 MAPK activation when compared to ventilation and LPS (Figure 22). In order to examine whether CO-induced p38 activation exerted biological effects, we measured the levels of the anti-

inflammatory molecule IL-10. We demonstrate that inhibition of p38 with the chemical inhibitor SB203580 compound significantly attenuates CO induced IL-10 levels (Figure 23) and histology shows hemorrhagic, inflamed lung tissue (Figure 17G).

5. Discussion

International analyses found that the incidence of ALI is 22.4 cases/100,000 population in developed countries (23-30) . This shocking number is even higher when number are looked at in intensive care units (ICUs) where the prevalence of ALI/ARDS is 5-15% (28). ARDS is the most severe form of ALI with a high mortality rate 40-50%(2). Approximately 10 to 15 percent of patients admitted to an intensive care unit and up to 20 percent of mechanically ventilated patients meet criteria for ARDS (29). According to an international study, an average of 39% of intensive care unit patients requires mechanical ventilation worldwide (73) . Many of these patients develop ventilator-induced lung injury (VILI)(74). Eventually VILI contributes to acute respiratory distress syndrome (ARDS) (2). While clinical trials showed that ARDS/VILI-related mortality could be attenuated with lower tidal volume ventilation, PEEP ventilation and more recently, with recruitment maneuver combined with protective ventilation strategy, the syndrome remains a major problem in intensive care units (52, 75).

5.1 Gene expression profiling of target genes in ventilator-induced lung injury (Specific aim #1)

Cumulating experimental and clinical evidence suggests that VILI is triggered by mechanical overstretch of the alveoli, frequently facilitated by other causes of acute lung injury such as microbial agents. Lipopolysaccharide (LPS), acid

aspiration and cecal ligation/perforation induced sepsis are commonly used models for approximating previous lung injury in ventilator-induced lung injury models(18, 76). However, the extent to which overstretch and microbial agents activate similar or separate intracellular mechanisms it is not yet established. I approached this problem by comparing the gene expression profiles of overventilated and LPS-treated mouse lungs. Gene expression changes in ventilator-induced lung injury can be key to understand the detailed mechanism of ALI/ARDS. However the application of this approach to ventilator-induced gene expression *in vivo* is complicated by at least two factors: (i) in intact, non-injured animals alveolar overdistension is limited by both the chest wall and adjacent alveoli, (ii) infiltrating inflammatory cells may alter the gene expression profile. These two problems are circumvented in isolated blood-free perfused lungs. Therefore, in the present study we have used microarray analysis to study the effect of overventilation on gene expression and compared this gene expression pattern to that induced by LPS. My colleagues and I identified 567 genes whose expression was modified by overventilation and/or LPS (Table 6,7). Many genes, in particular those that code for cytokines, growth factors and apoptosis-related proteins were altered both by overventilation and by LPS suggesting the presence of common intracellular pathways. However, the expression of some genes including growth factors, metabolizing enzymes, mediators and cytoskeletal proteins were modified by alveolar distention only. It is tempting to speculate that these genes activate mechanisms different from the well-known lung inflammatory pathways that have been established for microbial infections.

The largest group of genes affected by overventilation was related to apoptosis. Epithelial apoptosis has been observed in both *in vitro* and *in vivo* models of VILI (77, 78) The genes identified in this study include genes involved in the FAS

ligand and the caspase pathways. Among the affected genes, many are expressed in nervous system pointing to a possible neuro-inflammatory axis. For example, expression of the neuropeptide preprotachykinin-1 was related to acute lung in injury in a mouse VILI model (79). Also, Hayashi and colleagues have reported that in patients with spinal muscular atrophy, mechanical ventilation appears to contribute to neurodegeneration (80).

Validation of the data and comparison to previous studies

Microarray analysis generates a huge amount of data and hence validation is an important part of the analysis. In the present study the expression pattern of 5 genes was further substantiated by RT-PCR analysis and in addition that of amphiregulin on the protein level. Another form validation is possible by comparison to previous studies. The isolated perfused mouse lung model is well characterized and production of interleukin-6 (IL-6), tumor necrosis factor (TNF), GM-CSF (unpublished data), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), cytokine-induced neutrophil chemoattractant (KC) and macrophage inflammatory protein-2α (MIP-2α) in response to OV has been documented (76). According to the gene array the induction of these genes by OV in relation to controls was MCP-1 (Ccl2) 8.8-fold, MIP-1α (Ccl3) 6.6-fold, IL-6 5.3-fold, KC (Cxcl1) 2.9-fold, GM-CSF (csf2) 2.04-fold, and TNF 1.2-fold (MIP-2α was not present on the chip). Thus, the protein release data are well corroborated by the gene expression data, although due to the small number statistical significance was not reached in all cases. The low expression of TNF in the array is explained by the fact that this gene is only transiently expressed after OV and is already on the decline after 180 minutes.

So far, only two gene array studies on ventilator-induced gene expression have been published (81, 82) Grigoryev *et al*. analyzed lung tissue from mice ventilated *in vivo* for 2h with 15 ml/kg. Of the 69 genes for which they provide detailed data (their supplementary table 4), 62 were also detected by our analysis. The correlation for the mouse data between these two studies is highly significant (Rho = 0.57, p = 0.02) (Figure 24) While in both studies only few genes reached statistical significance if analyzed within the context of the array, taken together these two studies provide a robust set of genes induced by overventilation including amphiregulin, activation transcription factor 3 (ATF3), MCP-1 (Ccl2), tissue factor (F3), growth arrest and DNA-damage-inducible 45A (GADD45A), glutamate-cysteine ligase, heat shock protein A8 (HSP A8), IL-6, urinary plasminogen activator receptor and cyclooxygenase-2. Consistent down-regulation was observed for the adrenomedullin receptor, TCF21 (transcription factor 21) and TFF2 (recoil factor 2). Further studies on these proteins should provide deeper insight into the mechanisms of VILI.

Of interest is the observation that the correlation between the two mouse studies and the rat study was only poor. This may be explained either by the longer duration of the rat experiments (5h) or by species differences. Nonetheless, most of the robust genes listed above appear to be similarly affected by overventilation in rats. Copland and colleagues studied overventilated rats for 30 min in what is now sometimes called a macroarray (590 genes) (82). In accordance with our findings these authors observed increased expression of B-cell translocation gene 2 (Btg2) (upregulated 2.18-fold by OV), epidermal growth factor 1 (EGR1) (6.2-fold), and Nur77 (2.04-fold), adding these genes to the list of candidate genes for ventilator-induced lung injury.

Differences between overventilation and LPS

Thus a number of important target genes were recognized by comparing arrays from different studies. Another approach is the use statistical methods to identify likely candidate genes within a given study. Herein we have used the combined statistical power of Scoregene and SAM. Applied to our data 90% of the significant genes identified by these procedures were identical, summing up to 567 genes regulated by overventilation and/or LPS.

Among the genes upregulated by both overventilation and LPS (Table 7) figured well-known pro-inflammatory (IL-6, GM-CSF, MIF) and anti-inflammatory (IL-10, IL-1ra) cytokines. Noteworthy cytokines upregulated by overventilation, which are new to the field of VILI, are IL-11 (confirmed by RT-PCR), macrophage migration inhibitory factor (MIF) and IL-22 (IL10-related T cell-derived inducible factor). Other extracellular factors upregulated by overventilation are amphiregulin (see below in The potential role of Amphiregulin) and cysteine-rich protein 61 (Cyr61, confirmed by RT-PCR). Cyr61 binds to cell surface integrins and thereby induces intracellular signaling events, some of which relate to cell proliferation and angiogenesis (83). Moreover, we identified several factors involved in intracellular signaling pathways such as gravin (Akap12, A-kinase anchoring protein 12) and Nur77 (Nr4a1) to become activated by overventilation. Since these genes may point to relevant mechanotransduction pathways, we confirmed their expression by RT-PCR. AKAPs maintain multivalent signaling complexes by binding additional enzymes, including kinases and phosphatases to the cytoskeleton and may thus communicate cell distortion inside cells (84). Nur77, also known as NGFIB or TIS1, represents an orphan nuclear receptor that is involved in cell proliferation and death and may act as an anti-apoptotic factor (85, 86).

A selective response was observed for another set of genes that according to the microarray analysis was upregulated by overventilation but not by LPS (Table 6). This list includes Tnfrsf12a (Fibroblast growth factor regulated protein 2, Fn14), which modulates NF-κB activation and stimulates human bronchial epithelial cells to produce IL-8 and GM-CSF (87), the largely unexplored chemokine CXCL16, and the chemokine receptor CCR5. The majority of these factors has not yet been implicated in VILI and indicates the existence of unknown specific mechanotransduction pathways in VILI.

Also of note is the fact that we found that a number of genes were down regulated by overventilation. So far, gene regulation has been largely focused on upregulation of genes and it is only with the beginning of microarray experiments that downregulation gets some focus. Prominent among the genes downregulated by overventilation figure platelet-derived growth factor and hypoxia-inducible factor 1α (HIF1α).

5.2 The potential role of amphiregulin (a new candidate gene in ventilator-induced lung injury) (Specific aim #2)

Stretch can induce growth factors in epithelial cells (88, 89). Tschumperlin and colleagues demonstrated that epithelial stretch increases epidermal growth factor receptor (EGFR) ligand shedding into the lateral intracellular space leading to intracellular signaling. The ligands bind to the EGFR that subsequently induces ERK mitogen-activated protein kinase (MAPK) activation (89). This mechanism could explain why several growth factor-related genes exhibited modified expression during overventilation.

One of them is amphiregulin. It is a polypeptide growth regulator and part of the epidermal growth factor family (90). Together with other EGFR ligands

amphiregulin binds to the extracellular domain of EGFR (91). EGFR binding leads to the activation of the ERK and the transcription factor NFκB. The EGFR induces cell proliferation and the release of IL-8 and/or MIP-2 cytokines (92-94). Amphiregulin is expressed in lung tissue and has an essential role in lung branching morphogenesis(95). In human pulmonary epithelial cells amphiregulin secretion is induced upon exposure to tobacco smoke and fine particulate matter (94, 96). Furthermore, increased amphiregulin secretion also contributes to GM-CSF release (96). In a mouse model of chronic asthma, ovalbumin-challenged animals displayed increased amphiregulin immunostaining of epithelial cells (97). Our immunostaining shows stretch-induced expression of amphiregulin in epithelial cells, which are exactly those cells in which extracellular-signal regulated kinase (ERK) is activated during overventilation. The possibility that amphiregulin mediates some of the signaling responses during overventilation merits further investigation.

Taken together, this findings suggest that overventilation activates specific cellular programs that clearly distinguish it from LPS. In the future it will be important to further characterize these pathways, which may finally allow to specifically interfere with the side effects of mechanical ventilation(98).

5.3 Inflammation and cell damage in acute lung injury

To understand the mechanism of lung injury we have to gain better information on the role of the participating cells. Cellular injury and damage is a major source of inflammatory mediators in ALI (99). There is bench and clinical research evidence that alveolar cells and infiltrating inflammatory cells both contribute to the development of lung injury (22, 42, 100). Alveolar cells consist of Type I and II pneumocytes, alveolar macrophages and endothelial cells. Infiltrating

inflammatory cells are predominantly polymorphonuclear leukocytes. Pro-inflammatory cytokines released from epithelial and endothelial cells lead to inflammatory cell activation that contribute to further injury. The systemic activation of the inflammatory cascade can cause epithelial and endothelial injury through reactive oxidant species, proteases and cytokines. As a result of capillary leakage protein and fibrin-rich fluid leaves the vessels leading to activation the coagulation system. The secondary release of growth factors and profibrotic mediators cause not only the activation of a healing and repair response but also contributes to the ongoing inflammation and damage to the lung. The complex mechanism of cellular activation including intracellular mechanisms is shown in Figure 25.

1.The role of epithelium

The alveolar surface is lined with flattened type I cells while corners of the alveoli are covered with cuboid shaped type II cells. Alveolar epithelial cells suffer the most injury in ARDS. The result of epithelial damage is alveolar barrier rupture causing alveolar edema and pro-inflammatory cytokine release. Widespread alveolar epithelial damage and necrosis is reported to be found in the lung of patients dying with ARDS (101, 102).The alveolar epithelium is also a barrier that avoids the influx of bacteria from the alveolar space to the circulation. In experimental pneumonia and ventilator-associated pneumonia the damage of the alveolar epithelium leads to bacteremia, sepsis and MODS (15, 103). Type II cells produce both the protein and lipid components of surfactant. Surfactant function and metabolism is inhibited in patients with ALI/ARDS. The impaired surfactant function contributes to alveolar collapse and hypoxemia. Besides type II-cell damage serum proteins flooding the alveolar space can also contribute to surfactant inactivation. Another important role of the epithelium and especially type II cells is edema fluid clearance. Patients with

decreased edema clearance have higher mortality rate in ARDS (51). However many patients with ALI/ARDS have sufficient edema clearance. The exact etiology of impaired edema clearance is not yet understood. It seems that it is more related to the severity of epithelial injury and type II-cell injury. Surfactant protein-D (SP-D) is suggested as a marker of alveolar type II-cell injury. SP-D was found to be decreased in patients with ARDS when compared with controls. Low SP-D levels are associated with higher mortality in the ARDS patients but at present it is not known whether low SP-D levels have a pathologic consequence as well (4).

2.Endothelial damage

Endothelial injury is also a signature finding in ALI. Radiolabeled showed that microvascular permeability is increased in patients with ALI compared to those with hydrostatic pulmonary edema(104). Endothelin-1 (ET-1) a vasoconstrictor peptide is released in increased quantities from endothelial cells during injury. However it is difficult to tell if ET-1 is a marker if injury, if it has pro-inflammatory action or contributes to increased pulmonary vascular resistance in ALI (105). Von Willebrand Factor antigen (VWF) is also suggested to be biomarkers of endothelial injury. Studies concluded that patients at risk of ARDS more likely to develop the syndrome if their VWF serum level is increased (106).

3.Alveolar macrophages

Alveolar macrophages are thought to important sources of pro-inflammatory cytokines in ALI. Pugin and colleagues reported TNF-α and IL-6 production by macrophages when pretreated by LPS. These cells also produce IL-8 when exposed to cyclic stress(107). However these experiments were based on a macrophage transformed cell line (A549). Alveolar macrophages may therefore be an important stretch-responsive cell in the initiation of inflammation in ALI.

4.Specific inflammatory cells

Neutrophil leukocyte infiltration to the alveoli is the hallmark feature of ALI. ARDS patients have predominantly neutrophils in bronchoalveolar lavage fluid and the resolution of neutrophilia is a good prognostic marker in ARDS (108).Histologically, patients suffering from ARDS have a dramatic increase in pulmonary neutrophils as they are sequestered in the lungs. However patients with neutropenia can also suffer from ALI. This suggests that neutrophils are not necessarily required for the development of ALI.

During the inflammatory process neutrophils pass through the capillary bed to infiltrate the lung alveoli. The physical properties of both neutrophils and the vasculature play a significant role in the accumulation of inflammatory cells in the lungs. Pulmonary capillaries in general, are smaller in diameter than then neutrophils. Cells must deform to pass through the capillaries. The lungs act as a filter to slow neutrophil transit. In response to inflammatory stimulus the sequestration of neutrophils increases by nearly 5 times (109). This leads to the slugging of neutrophils in the pulmonary circulation. Neutrophils become less deformable due to the redistribution of actin from the central (g-actin) to the submembranous area (f-actin) preventing the cells form flattening. The increased influx of young forms of leukocytes from the bone marrow to the lung parenchyma creates a relative neutropenia in blood. The young neutrophils are less deformable and their trapping in the pulmonary vasculature contributes to the endothelial injury seen in ALI. Sequestred neutrophils arrive to the lung in an activated state with increased cytotoxic effects, reactive oxygen species (ROS) production, increased lysosomal enzyme release and increased expression of adhesion molecules (CD11/18) and decreased L-selectin expression. The activation is achieved by G-proteins coupled cytokines

receptors that transducer signals to nucleus (Figure 26). The known intracellular pathways that transmit these signals involve mitogen-activated protein kinase (MAPK). The MAPK pathway consists of multiple stepwise interacting cascades of serine/threonine phosphorylation. Extracellular signal-regulated kinase (ERK) family of MAPK are activated in neutrophils when stimulated and they contributes to oxidative burst, phagocytosis and apoptosis (110). Multiple cytokines and chemoattractants were found to contribute this process by stiffening of neutrophil membrane (111).These stiff neutrophils cross the endothelium with less success while they release large quantities of proinflammatory cytokines such as TNF-α and MIP-2. The increased activation also involves nuclear factors like NFκB and CREB. Their activation is also linked to ERK (112). While p38 MAPK is also believed to be involved in the modulation of neutrophil activation, there is confounding data whether this pathway is important in the regulation of the pathogenesis of ALI .

Initially within the pulmonary vascular bed, neutrophils adhere weakly. Following activation of the neutrophils and the endothelium various types of adhesion molecules are expressed on the surface of both cells. The intracellular mechanisms leading to the activation of neutrophils and endothelial cells is described in Figure 26. The expressions of adhesion molecules are a key feature of endothelial activation. Selectins are reactive oligosaccharides, a family of cell-cell adhesion molecules, expressed on neutrophils and endothelial cells. Integrins are transmembrane adhesion molecules that provide a second adhesion complex binding the neutrophil to the endothelium. Integrins are also capable of signal transduction, when bound can activate intracellular pathway mechanisms. The increased expression of intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), CD14, Tumor necrosis factor receptor II (TNFR II), E-selectin and P-selectin were

detected in ALI. Elevated levels of L-Selectin is observed in the beginning of the injury and declines with the attachment of neutrophils to the endothelium(113). Neutrophils themselves can regulate the activation of endothelium. Many cytokines, chemokines and the expression of adhesion molecules are regulated by transcriptional factors like NFκB (see below at Cytokines). Endothelial cell expression of adhesion molecules, such as ICAM-1, VCAM-1 and E-selectin are induced by pro-inflammatory cytokines and regulated by NFκB binding and translocation. This NFκB-dependent expression of endothelial adhesion molecules can be downregulated by antioxidants (114).Once neutrophils marginated they begin their diapedese across the endothelium and the interstitial space into the alveoli between the epithelial cells. This process also depends on the activated adhesion molecules.

Neutrophil leukocytes mediate lung injury in many ways. They release nitrogen and oxygen species, produces growth factors and cytokines and release proteolytic enzymes. Neutrophil elastase is the major released proteolytic enzyme. BALF analysis shows that patients at risk of ARDS and with ARDS have higher elastase levels in the lavage fluid. However most elastases are in complex with their natural inhibitors (α_2-macroglobulin, α_1- antitrypsin), which shows that neutrophil activation and degranulation is attenuated by the endogenous inhibitors. It is likely that the overall protease/antiprotease balance that determines the neutrophil-induced proteolytic damage (105).

Another key point in neutrophil caused inflammation is the dysregulation of apoptosis. Apoptosis is important in the withdrawal of inflammatory cells from the lung tissue. Apoptotic cells are taken up macrophages without causing damage to the tissue. However in ARDS, apoptosis is markedly reduced. A variety of neutrophil function inhibitors are found in the BALF of ARDS patients. The anti-apoptotic effect

is mainly mediated by G-CSF and GM-CSF, however neutrophil apoptosis correlates best with the level of anti-inflammatory cytokine IL-2.

5.Cytokines and chemokines

Cytokines and chemokines are extracellular soluble peptides that elicit biological activity through binding to cell surface receptors. They have paracrine and autocrine activity and they can be active in as low as 10^{-15}M concentration.

Several pro- and antiinflammatory cytokines are induced in ALI. A key aspect of this field is that cytokine balance is an important determinant of the duration and the degree of the inflammatory response. Early studies focused on finding prognostic cytokine mediators in ALI. Furthermore two major proinflammatory cytokines in ARDS, interleukin-1 β (IL-1β) and tumor necrosis factor -α (TNF-α) were targeted in a clinical setting without success (54). It is clear now that in the complex pathology of ARDS cytokines work in a network including pro and anti-inflammatory cytokines and inhibitors of pro-inflammatory cytokines. Another aspect of the networking of cytokines is that level of cytokine measured in BALF or in serum does not predict the biological activity of a cytokine. A better way to assess the net pro-or anti-inflammatory activity of a cytokine is by measuring its biological activity.

5.1 Proinflammatory cytokines IL-1β and TNF-α

Although many cytokines are implicated in the pathogenesis of ALI/ARDS, the key early mediators of inflammations are IL-1β, TNF-α. Both cytokines illicit similar proinflammatory activity are produced primarily by monocytes. These cytokines act in synergy on endothelium to induce the upregulation of adhesion molecules ICAM-1, P-Selectine, E-selectine. They also promotes pro-coagulant surface through the increase in tissue factor, reduction of surface thrombomodulin and increases the production of chemokines like monocyte chemotactic peptide-1 (MCP-

1), macrophage inflammatory peptide-1α (MIP-1α) and interleukin-8 (IL-8). The increased chemokine production shows chemotactic preference to neutrophils aiding their release from the bone marrow and priming them for cytotoxic effects (99).Of note, these cytokines were also tight to repair after lung injury in fibroblasts and epithelial cells(115).The increased IL-1 and TNF response can be linked to increased gene expression. The intracellular signal that leads to gene expression of these cytokines is regulated by DNA-binding proteins like the activation of nuclear-factor κB (NFκB) transcription factor. NFκB is bound to inhibitory κB (IκB) when not activated. Upon activation IκB degrades and NFκB enters the nucleus binding to promoter sequences on genes to enhance their transcription. Clinical and bench research data suggests that IL-1β and TNF-α is dependent on the activation of NFκB. In patients with ARDS NFκB and the cyclic AMP response element binding protein (CREB) were activated. While the exact mechanism of activation in the case of NFκB is not understood, the activation of CREB depends on xanthine oxidase (XO). XO a potent oxidant and it is linked to the generation of reactive oxygen species and the pathogenesis of ALI. CREB can be upregulated by LPS, cytokines and hypoxia (99).

5.2. IL-6

IL-6 is a product of IL-1β and TNF-α stimulated alveolar macrophages and it is found in high concentrations in the BALF of patients with ALI/ARDS. It serves to stimulate acute phase reactants (116). Soluble IL-6 receptor is also present in the BALF of ARDS patients and it functions to promote IL-6 signaling when bound to the gp130 receptor that is ubiquitously present on cell membranes. IL-6 signaling is mediated in part by mitogen-activated protein kinase (MAPK) signaling pathway, specifically through the phosphorylation of extracellular signal-regulated kinase 1/2

(ERK1/2) and p38. Inhibition of these pathways results in reduced in decreased production IL-6 (117).

Several other cytokines proinflammatory cytokines have been shown to enhance and prolong inflammation in ARDS including granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage inhibitory factor (MIF), transforming growth factor- β (TGF- β) and high mobility group-1 (HMG-1)(100).

5.3 Antiinflammatory cytokines

Interleukin-10 (IL-10) is an anti-inflammatory cytokine that has the ability to suppress proinflammatory cytokines in macrophages and its expression is closely related to TNF-α expression. It has been reported that IL-10 levels are increased in patients` lungs in ARDS and higher cytokine levels are associated with improved survival (118). The ratio of TNF-α/IL-10 has been reported to be elevated in patients with ARDS. However studies show that IL-10 levels peak at day 1 and decease over a 3 weeks period of time. These results suggest that in patients surviving ARDS over 3 weeks TNF-α/IL-10 ratio prefers a net antiinflammatory condition (100). Other candidates of antiinflammatory cytokines are IL-6 types cytokines: IL-11, cardiotrophin-1 (CT-1) and IL-6. They all share the common motive of gp130 receptor subunits. The anti-inflammatory and cytoprotective effects are mediated partly through the reduction of NFκB activation, the signal transducer and activator of transcription 3 (STAT3) and the mitogen-activated protein kinase-1 (MEK-1) pathways.

5.4 Chemokines

Chemokines are small polypeptides, related to cytokines, that are produced by a large variety of cells including endothelial and epithelial cells, fibroblasts,

monocytes and neutrophils via the action of proinflammatory mediators like LPS, TNF-α. and IL-1β. Chemokines cause leukocytes, especially neutrophils in ARDS, to change shape, follow a chemotactic gradient, promote adhesion to the endothelium and increase their cytotoxicity. Chemokines are divided into 2 groups according to the position of their conserved cystein residues. C-C chemokines have their cysteins adjacent to each other, in C-X-C chemokines the cysteins are separated by some other amino acid. An important subclass of these chemokines contains a glutamyl-leucyl-arginine (ELR) motif that is critical for neutrophil binding and chemotactic functions. Members of this subclass: interleukin-8 (IL-8), ENA-78, and GRO-α., Gro-β and GRO-γ are presented in biologically significant concentrations in BALF of patients wit ARDS. IL-8 is a key chemoattractant for neutrophils in ALI/ARDS. IL-8 levels are high in the BALF of patients with ARDS (100). Bronchial epithelial cells undergo apoptosis in response to TNF-α or Fas ligation. During this cells will produce IL-8. Fas ligation appears to regulate the production of IL-8and through the activation NFκB. Once IL-8 gene expression is activated the protein production is controlled by ERK 1/2 and p38 MAPK. Additionally TNF-α can activate p38 and increase IL-8 production. Antioxidants like N-acetyl cystein can reduce the TNF-α induced activation of p38 and downstream products like IL-8 (119). Despite the evidences of the role of IL-8 in the regulation of neutrophils, there is no correlation between the number of neutrophils and IL-8 levels in patient's BALF in ARDS. Recent studies have show that the binding of IL-8 to its binding protein, α-2 –Macroglobulin (α-2M) preserves its cytokine activity. This binding prevents IL-8 attachment to matrix proteins and facilitates neutrophil clearance by macrophage via α-2M receptors. Another C-C chemokine that is involved in the regulation of neutrophil chemotaxis are the macrophage inflammatory proteins (MIP). They are primarily secreted by

macrophages and their function depends on the activation and production of IL-1β and TNF-α. Regardless the time of the injury MIP levels are high in the BALF. The activation of MIP-1 and 2 follows a very precise pattern including neutrophil recruitment, increased vascular permeability and increased expression of adhesion molecules. Another potent chemoattractant is leukotriene B_4 (LTB_4) and platelet activation factor (PAF). The levels of these chemokines are elevated in ARDS and they serve to recruit neutrophils into the alveolar space. Both these mediators share the upstream enzyme phospholipase A_2 (PLA2). Plasma levels of PLA2 are significantly elevated in non-ALI septic shock and ALI/ARDS-related septicemia (120). A rabbit model of ALI have shown that the decreased activity of PLA2 is associated with less lung injury (121). The Duffy antigen is a minor blood group antigen that serves as receptor for many chemokines. There is evidence that it participates in the transport of chemokines across endothelial cells and therefore contributes to the regulation of inflammatory cell recruitment in tissue sites of inflammation (122).

6. Coagulation system

Patients suffering from ALI/ARDS have increased megacaryocytes and platelet counts. Fibrin microthrombi are commonly present in the extravascular pulmonary compartments in ARDS (123).A fine balance is present between the pro-coagulant and fibrinolytic pathways while in the inflamed lung pro-coagulant mechanisms primarily enhanced. Pro-coagulant pathways can influence cell trafficking, cell permeability and the activation of acute phase products. In normal lung tissue factor (TF) and factor VII (F VII) is abundant but the lung still maintains a fibrinolytic state due to the activity of urokinase plasminogen activator (uPA) and plasmin. In ARDS, these mechanisms are exhausted and fibrin rich hyaline

membranes form. Epithelial cells produce urokinase and its receptor (uPAR). uPAR and the leukocyte integrin MAC-1 have an important to promote fibrin degradation. IL-1β regulates uPA and the availability of intracellular iron. The intracellular decrease of iron downregulates uPA and upregulates the expression of plasminogen activiator inhibitor-1 (PAI-1) (124, 125). PAI-1 is important in the disruption of fibrinolytic activity. In addition, hyaluronic acid (HA) and thrombin is increased in ARDS. Despite the mounting evidence of the involvement of anti-fibrinolytic and systemic coagulation markers in ARDS there is no direct correlation between these factors and pulmonary damage(126). Recently, anticoagulant therapy for ARDS has shown positive results. Naturally occurring candidates are activated protein C (APC) that inhibits factor Va, VIIIa and PAI-1 and tissue factor pathway inhibitor (TFPI) that blocks factor VIIa (99).

Why can CO be used as an antiinflammatory agent?

In a rat model of ventilator-induced lung injury, I showed the aniinflammatory effects of low dose inhaled CO providing another evidence that this by product of heme degradation can be used to reduce lung injury *in vivo*.

1.Cytoprotection by HO-1: an overview

HO catalyzes the first and rate-limiting step in heme degradation. Tenhunen *et al.* first characterized HO as a microsomal monooxygenase system distinct from cytochrome p-450 (127). The HO enzymatic activity requires molecular oxygen and reducing equivalents from NADPH: cytochrome p450 reductase, to catalyze the oxidation of heme-b to biliverdin-IXα, which is further converted to bilirubin-IXα by an NAD(P)H-dependent reductase. The heme iron is liberated as Fe(II) as a consequence of heme cleavage, while the α-methene bridge carbon escapes as CO.

HO plays a principle role in degrading hemoglobin from senescent erythrocytes, in reticuloendothelial tissues such as the spleen, kidney, and liver. A schematic picture of heme degradation and the cytoprotective effects of its metabolites are shown in Figure 27.

HO exists in three genetically distinct isoforms (HO-1, HO-2, and HO-3). The transcriptional activation of the *ho-1* gene, and corresponding increases in protein and enzymatic activity, are stimulated in most cells and tissues by exposure to a wide spectrum of chemical and physical stresses, as well as physiological regulators, such as cytokines and growth factors. HO-2, the constitutively expressed isozyme, occurs at high levels in neuronal, vascular, testicular, hepatic, and other tissues. HO-2 does not respond to transcriptional activation by environmental stress, but may respond to hormonal regulation in the brain. Another constitutive isozyme, HO-3, which has little enzymatic activity, exhibits high sequence similarity with HO-2 and remains incompletely characterized. HO enzymes have been found in lower organisms as well. Variants of HO-1 have been described in bacteria, fungi, plants, and algae.

HO-1, the major 32-kDa mammalian stress protein, can be induced at the transcriptional level by multiple forms a cellular stress (128). Keyse *et al.* were among the first to suggest that HO-1 acts as a general cellular defense mechanism against oxidative stress, which has been demonstrated in cell culture (129) and in animal models of inflammatory or oxidative tissue injury (62). Numerous studies have linked HO-1 mediated cytoprotection to the biological activities of its enzymatic reaction products (130). Both biliverdin and bilirubin display considerable antioxidant properties *in vitro*. The HO-derived heme-iron regulates the synthesis of ferritin, an iron storage molecule, which acts as a cytoprotectant by sequestering and detoxifying the released iron (131). An intracellular iron pump associated with HO-1 has also

been associated with detoxification of the released iron (132). To further emphasize the importance of this enzyme, HO-1 gene deficient mice (*ho-1*$^{-/-}$) are born abnormal, die before the age of 1 year and suffer from various chronic inflammatory diseases. Endothelial cells derived from *ho-1*$^{-/-}$ mice display increased sensitivity to oxidative stress in culture. Similar disorders were shown in a unique HO-1 deficient patient described in 1999 (133).

2. CO confers antinflammatory, antiapoptotic and antiproliferative effects in vitro and in vivo

Carbon monoxide (CO) like nitric oxide (NO) arises endogenously in humans as products of ordinary metabolism. The variable occurrence of these gases in ambient inspired air, in combination with their systemic metabolic production which can increase during inflammatory disease states, contribute to their appearance at trace levels in the exhaled breath of humans (134). *In vivo*, both these diatomic gases originate from the enzymatic oxidation of organic precursor molecules. NO arises during the metabolic conversion of L-arginine to L-citrulline by nitric oxide synthases (NOS; E.C 1:14:13:39), while CO originates from the oxidative degradation of heme by the heme oxygenases (HO; EC 1:14:99:3). Furthermore, both enzyme systems consist of constitutive and inducible isozymes. The two gases differ however in inherent stability and reactivity. CO, which is much more stable than NO, typically reacts only with iron centers of heme-containing proteins. NO, a free radical, displays a broader spectrum of chemical reactivity, and has a much shorter lifetime in biological systems (135). Both NO and CO can occur in the environment as atmospheric pollutants, and are thus considered inhalation hazards. CO occurs ubiquitously as a product of the incomplete combustion of hydrocarbons. Common sources of CO include burning coal, wood, tobacco, and fossil fuels. Environmental

CO, when accidentally inhaled in poorly ventilated areas is a common cause of mortality. CO, especially dangerous due to lack of color, odor, or taste, can produce deleterious physiologic effects and, with prolonged exposure, can be lethal (136). The endogenous occurrence of CO has been known for approximately a half-century. CO normally exists in human blood tightly bound to the oxygen carrier hemoglobin. The majority of blood CO arises from endogenous erythrocyte degradation in the absence of significant environmental contamination. An estimated 86% of endogenous CO arises from endogenous heme metabolism, the majority of which occurs from hemoglobin turnover, with a minor component arising from the turnover of cytochromes and other hemoproteins in cells and tissues. Tenhunen and colleagues identified heme oxygenase as the principle enzyme involved in heme breakdown in 1968 (127). Since the discovery of the HO system, CO was widely regarded as an undesirable waste product of heme metabolism, and assigned little physiological significance. The remaining fraction of CO not associated with heme metabolism arises from poorly characterized sources, which may include the peroxidation of lipids, the photooxidation of organic molecules, and the metabolic conversion of xenobiotics (137). In 1998, the Nobel Prize in Physiology was awarded to Drs. Murad, Furchgott, and Ignarro for work leading to the discovery that the endogenous production of a similar small gas, NO could exert multiple physiological functions. Among these discoveries included the identification of endothelial derived relaxing factor, a substance involved in relaxation of vascular smooth muscle, as NO gas (138). These observations led to the realization that endogenously derived gases could participate in the regulation of physiological processes. NO produces its vasodilatory effect by binding to and activating soluble guanylyl cyclase (sGC), leading to the enhanced production of guanosine 3',5'-monophosphate (cGMP).

Snyder and colleagues, working with models of olfactory neurotransmission were among the first to propose that CO, could exert similar physiological functions as NO, also by acting on sGC (139). Verma *et al*. speculated on CO signaling pathways by observing a colocalization of HO-2 and sGC in distinct brain regions (140). The authors demonstrated an important role for cGMP in olfactory signaling in the central nervous system, and suggested the involvement of CO in this process by using inhibitors of HO activity.

Recent research has indicated that low concentrations of this gas may exert considerable vasoregulatory, anti-inflammatory, anti-apoptotic, and anti-proliferative activity in cell culture and in animal models, by influencing intracellular signal transduction pathways. Morita *et al*., demonstrated that CO can activate the sGC and cGMP system (sGC-cGMP) in vascular smooth muscle cells (SMC), which inhibits the proliferation of these cells (141). Otterbein *et al.* demonstrated an anti-inflammatory effect of CO in macrophages that depended on the cGMP-independent modulation of p38 mitogen activated protein kinase (p38 MAPK) pathways (60). In other models of CO regulation, however, such as the inhibition of SMC proliferation, links between sGC and p38 MAPK have now been described. The antiapoptotic potential of CO was first demonstrated in cell culture studies using fibroblasts or endothelial cells. Both the exogenous administration of CO or the over-expression of HO-1, inhibited TNFα-induced apoptosis in murine fibroblasts (142). In the endothelial cell model, the inhibitory effect of CO on TNFα-induced apoptosis could be abolished with the selective chemical inhibitor, SB203580, or a p38 MAPK dominant negative mutant, implying a critical role for the p38 MAPK pathway (143). Furthermore, HO-1 or CO co-operated with NFκB-dependent anti-apoptotic genes (c-IAP2 and A1) to protect against TNF-α mediated endothelial cell

apoptosis (144). In a model of intimal hyperplasia, where smooth muscle cells proliferate uncontrollably following balloon angioplasty of the carotid artery, exposure to CO also completely prevented stenosis of the vessel(145). Pre-treatment of a rat with CO (250 ppm) for just 1 h significantly reduced the neointimal proliferation seen at 14 days post-balloon angioplasty relative to control animals that did not receive CO treatment. The mechanisms involved in this effect were attributed to the inhibition of smooth muscle proliferation by CO. Smooth muscle cell (SMC) proliferation is regulated by the cyclin dependent kinase inhibitor $p21^{Waf1/Cip1}$. CO treatment induced $p21^{Waf1/Cip1}$ expression in SMC. The antiproliferative effect of CO was compromised in smooth muscle cells from the $p21^{Waf1/Cip1}$ knockout mice ($p21^{-/-}$). Although $p21^{Waf1/Cip1}$ is regulated by p53, the antiproliferative effect of CO occurred in SMC from p53 gene deficient mice ($p53^{-/-}$). CO treatment activated p38 MAPK in SMC. The antiproliferative effect and the upregulation of $p21^{Waf1/Cip1}$ by CO treatment depended on the activation of p38 MAPK, since these effects were reversed by the p38 MAPK inhibitor SB203580 (146).

3. Protective effects of CO in acute lung injury models

3.1. HO-1 and CO protect against hyperoxic lung injury

Mechanical ventilation with hyperoxia is commonly used in critical care medicine as supportive care for acute, severe respiratory failure. However, hyperoxia (>95% O_2) generates reactive oxygen species (ROS) that cause cell and organ injury. The damage occurs predominantly in the respiratory endothelium and epithelium. These targeted and damaged barriers can also represent sources of ROS that further exacerbate the injury. Hyperoxia, which evokes symptoms in mice similar to

human ARDS, causes cell growth arrest, cell death and inflammation in various *in vitro* and *in vivo* models (147). Exogenous CO, through anti-inflammatory action, protects the lung in a model of hyperoxia-induced lung injury (57, 62). Rats exposed to a low concentration of CO exhibit a marked tolerance to lethal concentrations of hyperoxia *in vivo*. This increased survival was associated with highly significant attenuation of hyperoxia-induced lung injury as assessed by the volume of pleural effusion, protein accumulation in the airways, and histological analysis. The lungs of rats receiving CO treatment (250 ppm) in combination with hyperoxia, were completely devoid of lung airway and parenchymal inflammation, fibrin deposition, and pulmonary edema relative to rats exposed to hyperoxia alone. Furthermore, exogenous CO completely protected against hyperoxia-induced lung injury in rats in which endogenous HO enzyme activity was inhibited with tin protoporphyrin, a selective inhibitor of HO. Rats exposed to CO also exhibited a marked attenuation of hyperoxia-induced neutrophil infiltration into the airways and total lung apoptotic index.

When mice were exposed to a hyperoxic environment (>98% O_2), they displayed signs of lung injury by 64-72 h, and 100% mortality within 90-100 h of exposure. The presence of CO (250 ppm) initiated prior to the hyperoxia, prolonged the survival of mice in the hyperoxic environment, increasing the LD_{50} to 128 h exposure. Similar to the results observed in the rat model, CO inhibited the appearance of histological markers of lung injury associated with hyperoxia, as well as markers of oxidative damage (ie. lung lipid peroxidation)(62). CO also inhibited the influx of neutrophils into the airways associated with hyperoxia treatment, as measured in bronchoalveolar lavage fluid (BALF). Hyperoxia induced the expression of pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6, by 84 h

of exposure and activated MAPK in lung tissue including ERK1/2, JNK, P38 MAPK and its upstream kinases MKK3/6. The protection afforded by CO treatment against the lethal effects of hyperoxia correlated with the inhibited release of the pro-inflammatory cytokine in the BALF.

MKK3$^{-/-}$ mice, or wild-type mice injected with the selective inhibitor of p38 α/β MAPK (SB203580), displayed the accelerated appearance of tissue damage markers (with the exception of neutrophil influx) and increased susceptibility to the lethal effects of hyperoxia, relative to wild-type mice. Cytokine mRNA (TNF-α, IL-1β and IL-6) expression in response to hyperoxia appeared earlier in the MKK3$^{-/-}$ mice relative the wild-type mice exposed to continuous hypoxia. CO did not inhibit the expression of the pro-inflammatory cytokines in the MKK3$^{-/-}$ mice, and furthermore did not confer protection or extend survival against hyperoxia in MKK3$^{-/-}$ mice or in wild-type mice injected with SB203580. In contrast, JNK$^{-/-}$ mice responded like wild-type mice with respect to the anti-inflammatory effects of CO. The protective effects of CO in this model were also observed *in vitro.* CO treatment of A549 lung epithelial cells increased the activation of MKK3, and specifically the β-isoform of p38 MAPK while suppressing that of the α-isoform. CO exposure increased the survival of A549 cells grown in continuous hyperoxia, relative to cells exposed to hyperoxia alone. Treatment with the SB203580 or transient transfection with dominant negative mutants of p38β or MKK3 abolished the cytoprotective effect of CO against hyperoxia. In summary, these experiments demonstrate that CO protects against the lethal and inflammatory effects of hyperoxia *in vivo* and *in vitro*, by downregulating the expression of pro-inflammatory cytokines, through a mechanism dependent on activation of the p38β, MKK3 pathway (62).

3.2. CO protects against ischemia/reperfusion injury

Ischemia-reperfusion (I/R) during lung surgery, lung transplantation, after hemorrhagic or cardiogenic shock often leads to tissue I/R injury. The massive cell death associated with I/R limits therapeutic options. Animal models have suggested that apoptosis is a major cause of cell death following I/R trauma in lung, heart, kidney and brain (148, 149) . Anti-inflammatory effects of CO have been demonstrated in models of I/R injury of the heart, lung, kidney, and small bowel (150). CO protected against liver I/R injury via activation of the p38 MAPK (151). Homozygous *ho-1* null mice (*ho-1*$^{-/-}$) displayed increased mortality in a model of lung I/R injury. ICO inhalation (1,000 ppm) partially compensated for the HO-1 deficiency in *ho-1*$^{-/-}$ mice, and improved survival following I/R (152). In this model, the authors proposed that the protection provided by CO involved the enhancement of fibrinolysis, by the cGMP-dependent inhibition of PAI-1, a potent smooth muscle cell proliferation activator produced by macrophages. Mice treated with an sGC inhibitor, ODQ, were not protected from I/R-induced lethality by CO. Independent investigations, also using the mouse lung I/R model, also demonstrated that CO exposure protected against I/R induced lung injury. A left hilar clamp was placed on mechanically-ventilated rats for 30 minutes. After removing the clamp, a two-hour reperfusion was allowed. CO was introduced through the ventilator for a 1 h pretreatment period and throughout the experiment. TUNEL staining showed decreased number of apoptotic cells in the lungs of CO treated animals. Chemical inhibition of p38 MAPK activity, or deletion of MKK3 as in *mkk3*$^{-/-}$ mice, abolished the anti-apoptotic effects of CO during I/R by preventing the modulation of caspase-3 activity. Exogenously applied CO at concentrations starting at 15 ppm, inhibited I/R-induced apoptosis in pulmonary artery endothelial cell (PAEC) cultures, associated with the CO-dependent activation of the p38β MAPK isoform and its upstream

MAPK kinase (MKK3), with concomitant suppression of ERK and JNK activation. Inhibition of p38 MAPK with SB203580 abolished the cytoprotective potential of CO in this model. The anti-apoptotic effect of CO also involved inhibition of Fas/FasL expression, and other apoptosis-related factors including caspases (-3, -8, -9) mitochondrial cytochrome-c release, Bcl-2 proteins, and poly (ADP-ribose) polymerase (PARP) cleavage (147, 151). These studies confirmed a link between p38 MAPK and the down regulation of caspase-3 activity by CO, as previously described for Fast-mediated apoptosis. In addition to I/R, apoptotic pathways play a central part in many models of disease. A better understanding of cytoprotection provided by CO *in vivo* could lead future therapeutic solutions in other illness.

5.4 Carbon monoxide confers antiinflammatory effects in ventilator-induced lung injury (Specific aim #3 and #4)

The primary goal of the *in vivo* mechanical ventilation study was to test the hypothesis that inhaled CO can confer protective effects in an animal model of VILI. I used intravenous LPS injection and/or a relatively injurious ventilator setting to induce lung inflammation, in ventilated animals in the presence or absence of inhaled CO. A protective effect of CO was observed. Then I describe the potential mechanism by which CO confers anti-inflammatory effects against VILI.

I used a relatively injurious VILI model in rats, which resulted in lung injury that features inflammation, and edema as assessed by cell count, TNF-α production, and protein in the BALF. I performed TUNEL assay in our lung tissues and did not observe evidence of cell death after VILI (data not shown). To better mimic a human disease course and maximize VILI, we used a sub lethal dose of LPS to prime and supplement ventilator induced lung injury, as often used by investigators (8). We

measured blood gas and arterial blood pressure parameters and did not observe significant changes in blood pH, pCO_2, pO_2 and arterial blood pressure (Table 5).

We initially observed a robust induction of HO-1 mRNA and protein in this model of VILI (Figure 21). Our laboratory and others have shown that HO-1 induction in response to cellular and tissue stress, *in vitro* or *in vivo*, is not only a reliable marker of cellular injury but also a physiologic response to defend against the inciting stress or cellular insult (57). Thus, in view of our observation that HO-1 was markedly increased in VILI, we sought to assess whether CO could be responsible in mitigating VILI.

Using the same concentration of CO (250 ppm) we have used previously for *in vitro* and *in vivo* studies, we observed that CO could markedly attenuate the inflammatory responses of VILI. Inhaled CO significantly reduced the BALF cell count and TNF-α levels. Interestingly, we also observed that CO increased levels of the anti-inflammatory IL-10 in the BALF. These results correlate with the previous observations of Otterbein and colleagues in mice and murine macrophages (60).

Macrophages are the principal cell type found in the BALF after LPS/ventilation treatment. Macrophages are known to be sensitive to mechanical stress; thus, the changes in BALF total cell count might reflect an important role of macrophages in cytokine release in VILI (107) (also see above in *Inflammation and cell damage in acute lung injury* section). Belperio and colleagues described neutrophil leukocyte-predominant inflammatory response in mice following six hours high-volume (12ml/kg) mechanical ventilation (22) (also see above in *Inflammation and cell damage in acute lung injury* section) . In my model neutrophil leukocyte infiltration to the alveolar space was observed after 4 hours of mechanical ventilation. Inhaled CO significantly reduced neutrophil recruitment to the alveoli. Additionally,

inhaled CO also reduced BALF macrophage number at 2 hours. TNF-α is a well-known and well-investigated cytokine that has a pro-inflammatory effect in *in vivo* and *in vitro* models (76, 153) and IL-10 has anti-inflammatory activity in LPS-induced inflammation (154). Of note, it is interesting to note that CO did not affect the BALF protein levels suggesting that CO exerts negligible effects on pulmonary permeability. This observation provide us additional clue as to the differential and specific anti-inflammatory effect of CO which at this time appears to act as a regulator of inflammation by attenuating pro-inflammatory cytokine and augmenting anti-inflammatory cytokine.

The signaling pathway, by which CO acts as an anti-inflammatory agent, is not fully understood. Although it is well established that CO activation of sGC and cGMP mediates much of the vasodilatory effects (155), we did not observe a cGMP dependent effect in our VILI model (data not shown). We showed that NF-κB and AP-1 activation, two major pathways in ventilator-induced lung injury (8, 76), are not modulated by CO inhalation in VILI (Figure 23A,B). The p38 MAPK is known to regulate TNF-α and IL-10 production (156). Although the molecular mechanism by which CO affects p38 MAPK to produce less TNF-α and more IL-10 needs to be further investigated, CO may have a posttranscriptional effect on TNF-α production (Figure 24,25) (145).

I believe that our models can lead to a better understanding of the complex intracellular regulatory function of CO in lung injury. Based on the observations of this study, it is tempting to speculate that inhaled CO could represent a potential new therapeutic modality for counteracting VILI.

6. Summary

Acute lung injury (ALI) is a common and severe disease providing 5-15 % of all intensive care unit patients. Acute lung injury is often presented in its most advanced form, acute respiratory distress syndrome (ARDS) that has 40-50% mortality. Despite intensive investigation the mechanism of ALI is not well understood and its therapy is poor.

ALI was assessed in animal models where bacterium polysaccharide and mechanical ventilation was used to induce injury. Microarray profiling of mouse lung genes following injury lead to description of new target genes. The results suggest their important role in the mechanism ARDS. Major pathologic features of ARDS including alveolar edema formation, epithelial barrier disruption and inflammatory cell infiltration were described in an *in vivo* model. I showed the increased release of proinflammatory cytokine tumor necrosis factor-α and the induction of oxidative stress-inducible, heme oxygenease-1 enzyme following mechanical ventilation. Neutrophil leukocyte infiltration and proinflammatory cytokine levels in the bronchoalveolar lavage fluid were attenuated with low dose inhaled carbon monoxide (CO) while antinflammatory cytokine interleukin10 levels increased. CO has anti-inflammatory effects in other lung injury models as well but the intracellular mechanism is only partly understood. I showed that CO exerts its effects independent of two major inflammatory pathways nuclear factor-κB and activator protein-1 but involving p38 mitogen-activated protein kinase in the model.

My findings support the theory that inhaled carbon monoxide can reduce lung inflammation suggesting a new therapeutic approach to ALI/ARDS-related morbidity. I would like to continue further investigation of the applicability of CO in ALI hoping that one day it can be used in medical intensive care.

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Acknowledgements

First, I would like to thank my mentors for continuous support and encouragement;

To Augustine who taught me what makes a good scientist, invested time and energy into me and shared his outstanding view of the world and science, To Ildikò, who introduced me to international world of Pulmonary medicine and helped in a million way, To Marika, who gave me a sense what it is to be physician and taught me to care about the patient above all.

To Mingyao Liu who accurately corrected my research work and shared the latest updates of science, To Jòzsef Balla who took my research ideas seriously and help all along the way in Debrecen, To Hong P. Kim "Dr.Kim" who taught me that patience is the power of science, To Judith Sarady for helping me to learn basic techniques in the lab, To Stefan Ryter for long hours gelshifting with me, To Leo Otterbein for his excellent ideas ,To every single member of "Choi lab" who made Pittsburgh my home, To Paul Reynolds for his help with Taqman and for the great conversations, To Donna Stolz and Tim Oury for all the histology work, To my parents who trust me more than I will ever trust myself., To my sister for encouraging my adventurous side, To Naftali Kaminski for sharing his knowledge of microarray, To Stefan Uhlig who let me work with his model and hosted me in Germany, To Sören Pischke to be a great friend and fellow scientist, To Anne Germain for the teaching me how to live like an intellectual and work like a dog, To Rajesh Pandav who taught me to accept people as they are and that SPSS is not very difficult, To Jenn Baldwin correcting my English and my life.

Table 1

Most common conditions associated with acute lung injury

Sepsis
Aspiration
Infectious pneumonia
Severe trauma
Surface burns
Multiple blood transfusions
Leukoagglutinin reactions
Pancreatitis
Drug overdose
Near drowning
Smoke inhalation
Cardiopulmonary bypass
Pulmonary contusion
Multiple fractures
Following upper airway obstruction
Following bone marrow transplantation
Drug reaction
Venous air embolism
Amnionic fluid embolism
Neurogenic pulmonary edema
Acute eosionophilic pneumonia
Bronchiolitis obliterans organizing pneumonia (BOOP)
Miliary tuberculosis

Table 2 Subgroup analysis of etiology of patients with acute lung injury in a European ICU study (from reference #11)

	Patients with ICU stay 24 h or more, or ALI/ARDS on admission (*n*=3511)	ICU patients with ALI or ARDS (*n*=463)
Age (years) mean (SD)	59.4 (18.4)	54.7 (18.4)
Male sex, *n* (%)	2,111 (60.1)	296 (63.9)
Admission category, n (%)		
Surgical	1,567 (44.8)	162 (35.1)
Medical	1,935 (55.2)	299 (64.9)
Trauma, n (%)	598 (17.0)	102 (22.0)
Chest trauma	65 (1.9)	65 (14.0)
Chronic organ failure, *n* (%)		
One or more	900 (25.6)	103 (22.2)
Respiratory failure	409 (45.5)	32 (6.9)
Cardiac failure	312 (34.7)	25 (5.4)
Liver failure	115 (12.8)	25 (5.4)
Renal failure	120 (13.3)	8 (1.7)
Haematological disease	6 (8.4)	27 (5.8)
Immuno-incompetence	451 (12.9)	91 (19.7)
Acute organ dysfunction on admission, *n* (%)		
Number/type of organ dysfunction		
0	229 (6.5)	8 (1.7)
1	621 (17.7)	31 (6.7)
2	2,661 (75.8)	424 (91.6)
Respiratory	2,794 (79.6)	442 (95.5)
Cardiovascular	1,376 (39.2)	246 (53.1)
Neurological	1,057 (30.1)	170 (36.7)
Renal	2,289 (65.2)	341 (73.7)
Liver	318 (9.1)	74 (16.0)
Haematological	254 (7.2)	80 (17.3)
Gastro-intestinal tract	110 (3.1)	17 (3.7)
Mechanical ventilation, *n* (%)	2768 (78.9)	439 (94.8)
SAPS II on admission, median (IQR)	35 (24–45)	41 (31–51)
Length of stay, mean (SD)		
ICU	9.0 (12.4)	16.4 (15.8)
Hospital	22.1 (26.0)	25.8 (27.7)
Mortality rate, *n* (%)		
ICU[a]	628 (18.0)	210 (45.8)
Hospita [b]	904 (27.5)	234 (54.7)

Table 3 Phases of diffuse alveolar damage (from reference #35)

Exudative (days 1-7)	Proliferative (days 7-21)	Fibrotic (> day 21)
Interstitial and intraalveolar edema	Interstitial myofibroblast reaction	Collagenous fibrosis
Hemorrhage	Lumenal organizing fibrosis	Microcystic honeycombing
Leukoagglutination	Chronic inflammation	Traction bronchiectasis
Necrosis Type I cells Endothelial cells	Parenchymal necrosis	Arterial tortuosity Mural fibrosis Medial hypertrophy
Hyaline membranes	Obliterative endarteritis	
Platelet-fibrin thrombi	Macrothrombi	
Increased megakaryocytes		

Table 4
Methods commonly used in the diagnostics of acute lung injury an acute respiratory distress syndrome

Method	Advantage	Disadvantage
Bronchoalveolar lavage	Can be done at any time during the course of the disease Can be done in at-risk patients Can sample the alveolar cell population	Variable dilution factor Invasive Not feasible if severe hypoxia is present
Pulmonary edema fluid sampling	No dilutional factor Simultaneous edema fluid and plasma levels of mediators can be compared Relative noninvasive Can be used to calculate rate of alveolar fluid clearance	Only feasible when alveolar flooding is present Not all patients will have aspirable edema fluid
Blood sampling	Relatively noninvasive Can be done at any time during the course of the disease	Samples only the intravascular compartment
Exhaled breath condensate	Completely noninvasive Can be done at any time during the course of the disease	Source of exhaled molecules unclear (nasopharyngx, airway, alveolus) Evaporation may affect concentrations Relationship to alveolar lining fluid unclear
Exhaled gas analysis	Completely noninvasive	Only useful for volatible compounds May be technically difficult
Imaging	Completely noninvasive	Patient transport may be required
Chest radiography, CT, PET scan	Provides information about distribution of disease not available via other modalities	Relatively descriptive
Extravascular lung water measurements	Provides better quantification of degree of pulmonary edema than chest radiograph Can be used to guide fluid management	Does not reliably distinguish between hydrostatic and increased permeability pulmonary edema Invasive May be confounded by high cardiac output
Lung microvascular permeability	Can quantify protein permeability of the alveolar capillary barrier Relatively noninvasive	May require patient transport Findings differ depending on tracer used
Wasted ventilation	Completely noninvasive Quantifies degree of lung microvascular destruction/obstruction	Does not differentiate cause of lung microvascular destruction/obstruction
Genetic studies	Has potential to identify genetic predispositions for the development of ALI/ARDS	Given the large number of factors that probably determine whether someone develops ALI, it may be difficult to pinpoint specific mutations that increase risk
Lung biopsy	Allows histological and ultrastructural analysis at various timepoints during ALI/ARDS	Very invasive Rarely done for diagnosis
Autopsy	Allows histological and ultrastructural analysis of the entire lung	Samples only sickest patients who die from the illness Not doe routinely

Table 5 Mean arterial blood pressure, blood gases and carboxy-hemoglobin (CO-Hbg) prior and after mechanical ventilation with and without CO

	mean arterial BP (mmHg)		pH		PCO_2 (mmHg)		PO_2 (mmHg)		COHb (%)	
	start	end	start	end	start	end	start	end	start	end
LPS/ vent.	82±5	118±6	7.34± 0.06	7.22± 0.05	22±5	40±3	104± 10	86± 12	**6.5± 1.2**	**6.2± 0.8**
LPS/ vent./ CO	84±3	122±4	7.32± 0.02	7.26± 0.03	23±4	46±5	108± 18	90± 19	**5.8± 1.7**	**14.5± 2.1**

Table 6 Genes regulated only by overventilation

Fold change units: log_2ov/c

GeneBank ID	Gene name	Fold change	GeneBAnk ID	Gene name	Fold change
NM_013749	FGF regulated protein 2	3.42	NM_013685	Transcription factor 4	-2.88
AA105755	Na/K transporting ATPase	1.13	NM_008808	Platelet-derived growth factor	-1.33
NM_017472	Sorting nexin	1.16	NM_008929	Protein kinase, IFN-inducible	-2.38
NM_011345	Selectin (endothelial cell)	0.82	NM_008483	Laminin β2	-1.84
NM_01080	Matrix metalloproteinase 3	2.09	NM_010431	Hypoxia-inducible factor 1α	-2.49
NM_023158	CXC chemokine ligand 16	1.15	NM_007637	Chaperonin subunit 5	-2.01
NM_009941	Cytochrome C oxydase	0.77	NM_007512	ATPase inhibitor	-2.13
NM_019549	Pleckstrin	1.189	NM_009429	Tumor protein, transl. controlled	-2.26
NM_009917	Chemokine (C-C) receptor 5	1.26	NM_007610	Caspase 2	-2.3
NM_010637	Kruppel-like factor 4	1.36	NM_010406	Hemolytic complement	-2.64
NM_011607	TenascinC	1.86	NM_009779	Complement component 3A	-1.33

Table 7 Genes regulated by overventilation and LPS

Fold change units: log_2ov/c

GeneBank ID	Gene name	Fold change
NM_019713	RAS-associated domain family 1	3.14
NM_008655	GADD 45 B	2.88
NM_010798	Macrophage migration inhib.factor	0.89
NM_009805	CASP8 and FADD-like apoptosis regulator	2.31
NM_009704	Amphiregulin	3.19
NM_031167	IL-1 receptor antagonist	1.8
NM_010516	Cystein rich protein 61	1.63
NM_031185	A kapl 2	3.3
NM_010444	Nur77	1.42
NM_011337	Small inducible ctokine A3	2.73
X02333	GM-CSF	1.4
NM_031168	Interleukin 6	2.21
NM_008350	Interleukin 11	2.01
NM_016971	IL10-related T cell derived inducible factor	2.3
NM_010548	Interleukin 10	1.19

GeneBank ID	Gene name	Fold change
NM_021420	serin/threonin protein kinase 4	-2.01
NM_013546	Heme binding protein 1	-1.29
AF064749	Type VI collagen α subunit	-3.5
NM_022435	Trans acting transcription factor	-1.7
NM_009697	Nuclear receptor NR 2F2	-3.76
NM_010406	Hemolytic complement	-2.64
NM_033322	Small inducible cytokine family B	-2.99
NM_009069	RAS-like protein RIT	-2.7
NM_007993	Fibrillin 1	-1.53

Table 8 List of cellular functions with gene clusters, corresponding p-values and the number of statistically significant genes related to a given cellular function from 5612 valid genes

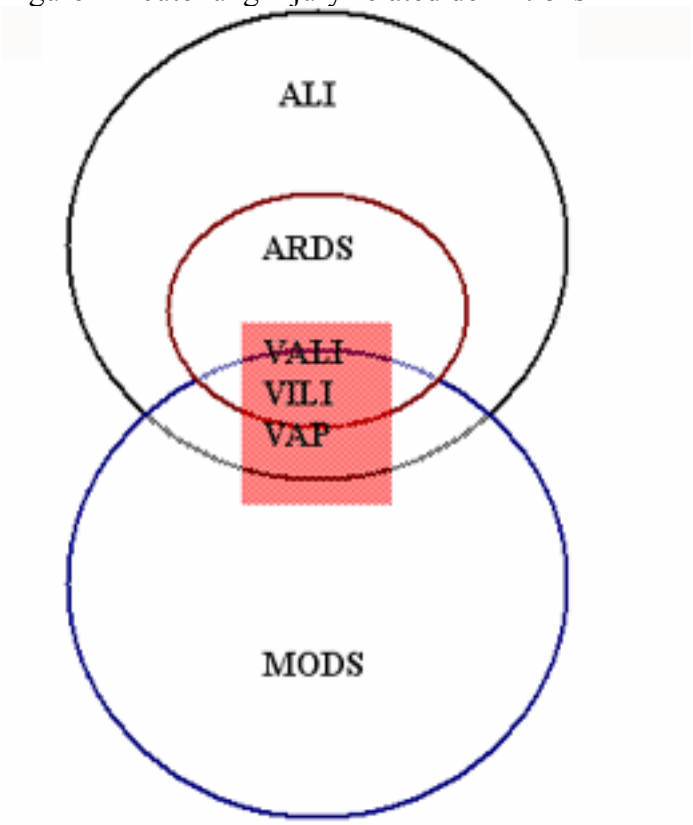
cellular fuction	cluster	p-value	number of genes	cellular function	cluster	p-value	number of genes
endomembrane system	up lps/c	4.78E-07	409	translation	up lps/c	0.01	211
endomembrane system	down ov/lps	4.62E-07	382	apoptosis	up lps/c	4.34E-04	62
nucleus	up lps/c	4.34E-08	407	apoptosis	down ov/lps	5.23E-03	88
nucleus	down ov/lps	8.65E-08	379	ribosome	down ov/c	4.60E-04	55
binding	up lps/c	4.00E-06	630	chromosome	down ov/c	4.74E-04	38
binding	down ov/lps	5.58E-07	592	chromosome	down ov/lps	3.73E-06	42
regulation of transcription	up lps/c	2.46E-04	230	mitochndrion	down lps/c	2.93E-03	135
regulation of transcription	down ov/lps	6.10E-06	223	nucleotide binding	up lps/c	6.36E-04	227
response to stress	up lps/c	1.52E-05	230	signal transducer activity	up lps/c	1.13E-04	603
response to stress	down ov/lps up	1.36E-03	206	carrier activity	up ov/c	3.85E-04	183
cytoskeleton	up ov/c	0.02	87	carrier activity	up lps/c	1.03E-04	207
cellular communication	up ov/c	8.15E-04	291	protein binding	up ov/c	1.25E-04	226
immune response	up ov/c	6.11E-03	162	protein binding	up lps/c	2.48E-04	414
immune response	up lps/c	2.7E-05	162	physiological process	up ov/c	3.38E-04	1084
cytokine activity	up lps/c	4.36E-03	46	physiological process	up lps/c	9.82E-13	1282
translation factor activity/n acid binding	up lps/c	4.13E-03	28	physiological process	down ov/lps	1.59E-08	1162

Table 9 Total and differential cell count in BALF following LPS treatment and 2 or 4 hours of mechanical ventilation with and without carbon monoxide (CO)

	Total cell number ($*10^4$ cells/ml)	Macrophages ($*10^4$ cells/ml)	Neutrophil leukocytes ($*10^4$ cells/ml)
Control 2 hours	2.11± 0.5	2.08± 0.31	0±0
Control 4hours	2.34± 0.66	2.31± 0.33	0±0
LPS/ventilation 2 hours	4.235± 0.43	4.192±0.21*	0±0
LPS/ventilation 4 hours	19.1± 1.8‡	16.32± 1.51‡	2.78± 0.27‡
LPS/ventilation/CO 2hours	2.9± 0.132	2.87± 0.06†	0±0
LPS/ventilation/CO 4 hours	13.75± 2.0§	13.51± 2.84	0.23± 0.17§

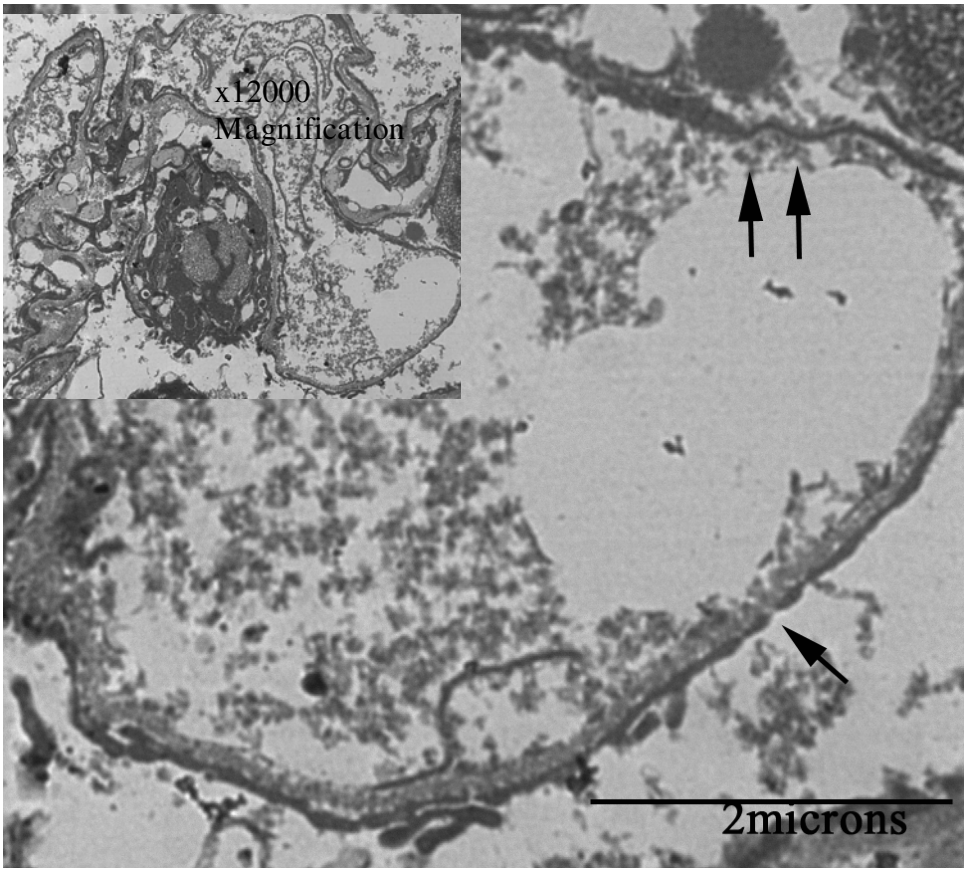
*p<0.03 LPS/ventilation 2 hours versus Control 2 hours condition, † p<0.029 LPS/ventilation/CO 2hours versus LPS/ventilation 2hours condition, ‡ p<0.01 LPS/ventilation 4 hours versus Control 4 hours condition , § p<0.05 LPS/ventilation/CO 4hours versus LPS/ventilation 4 hours condition, n=3 animals/condition

Figure 1 Acute lung injury-related definitions



ALI-acute lung injury
ARDS-acute respiratory distress syndrome
MODS-multiple organ dysfunction syndrome
VALI-ventilator-associated lung injury
VILI-ventilator-induced lung injury
VAP-ventilator-associated pneumonia

Figure 2 Elecromicroscopic picture of diffuse alveolar injury in mouse lung (picture T. Dolinay, D. Stolz)



legend: ↑ Damaged alveo-epithelial barrier

↑↑ Denuding epithelial layer adjacent to alveolar hyaline

Figure 3 Edema in the alveolar septi in acute lung injury in rat (picture T. Dolinay, H.P. Kim)

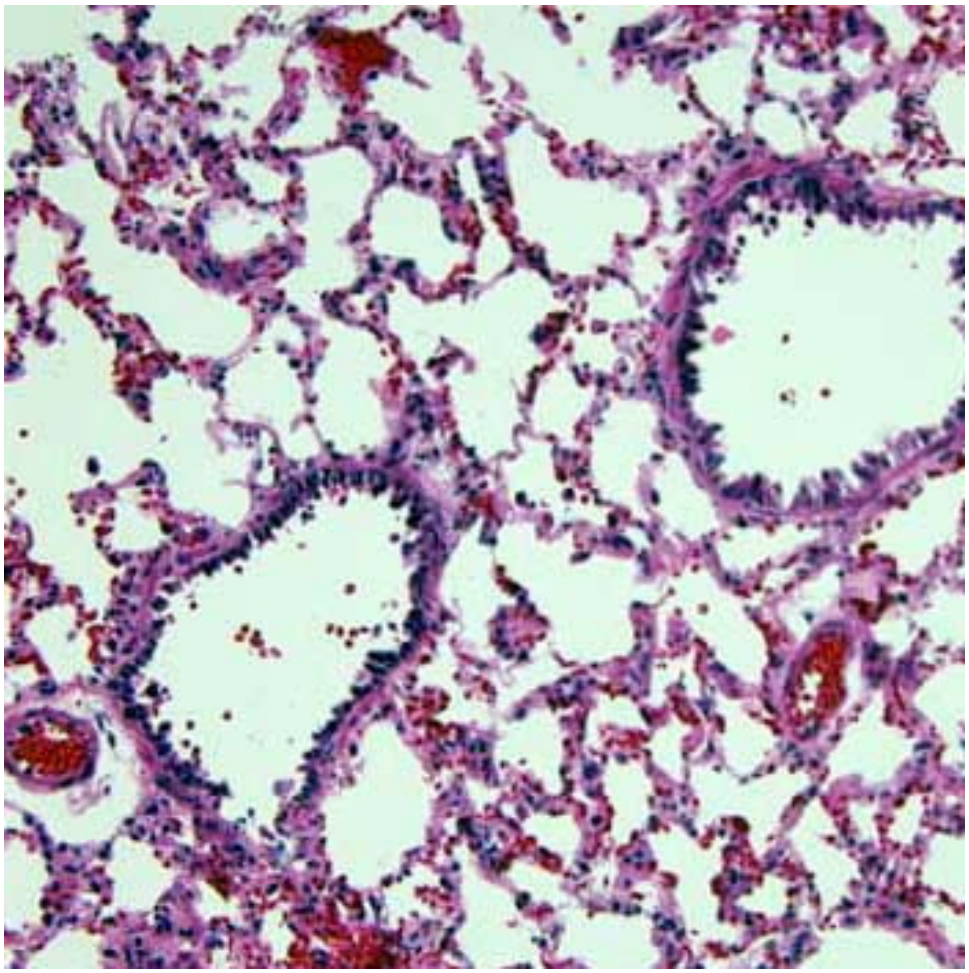


Figure 4 The potential mechanism of ventilator-induced lung injury (modified after Frank J.A., Matthay M.A. Critical Care 2003,7:233-241)

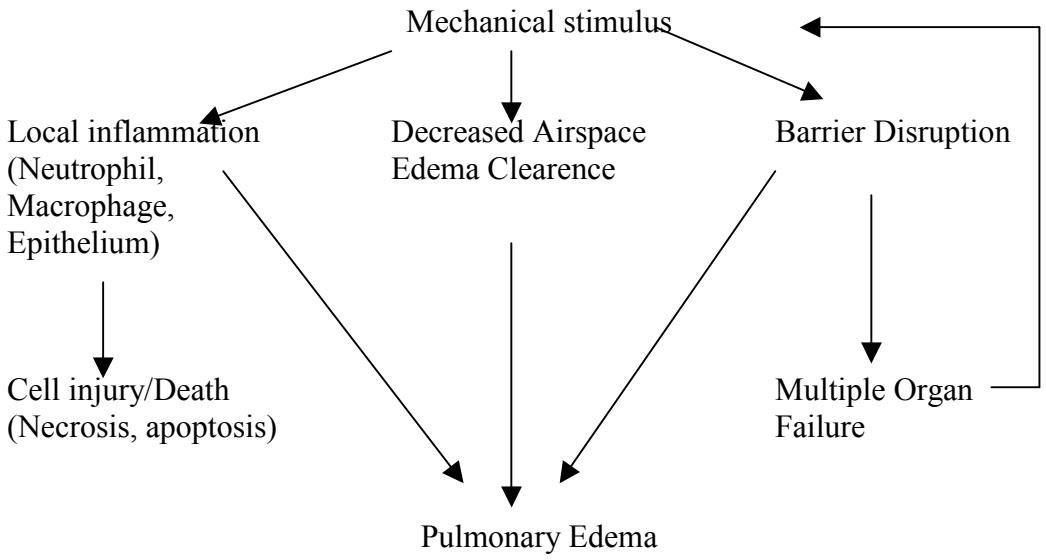


Figure 5. When alveolar cells are submitted to cyclic stretch proinflammatory cytokine production is observed (from reference #42)

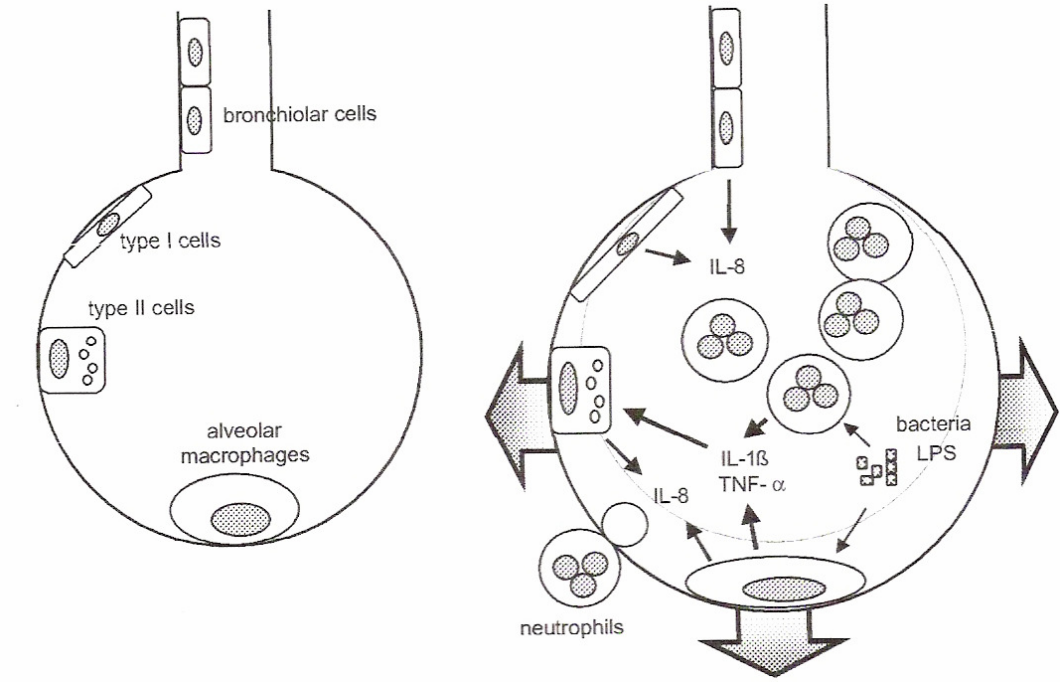


Figure 6. Isolated, perfused and ventilated mouse lung model (from reference #21)

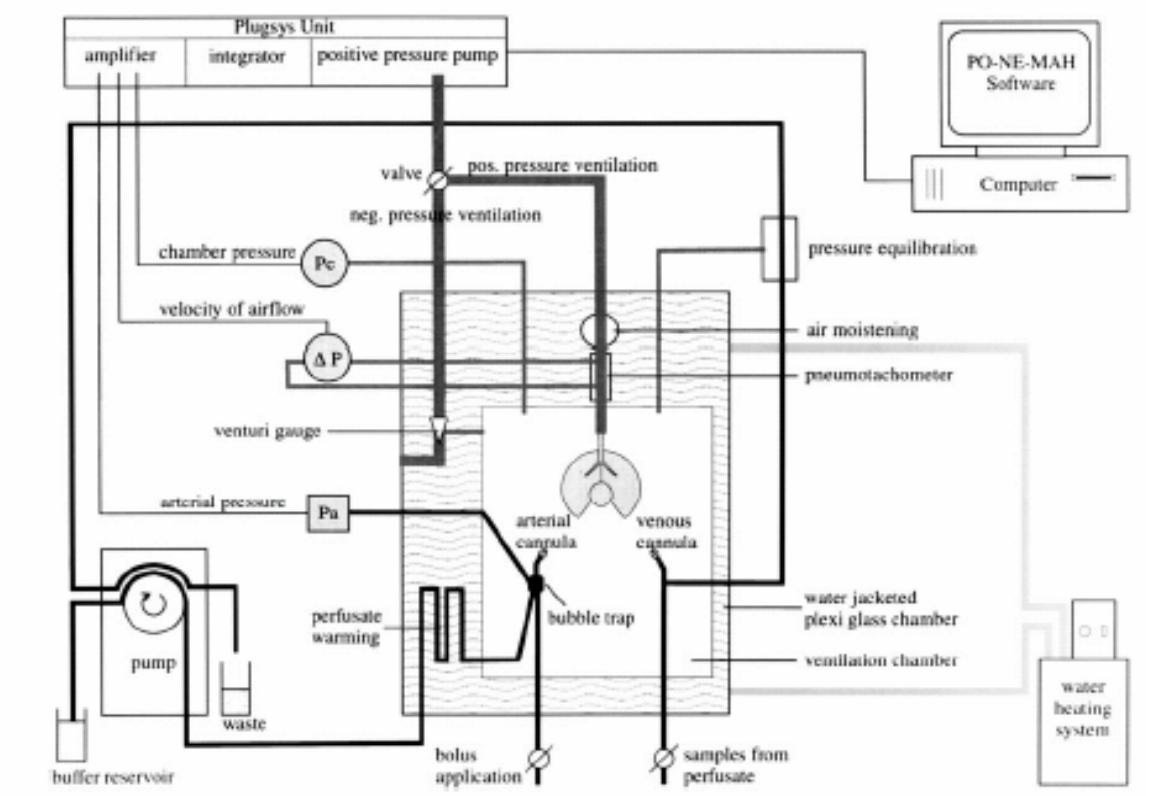


Figure 7. Experimental conditions in a rat model of ventilator-induced lung injury (from reference #63)

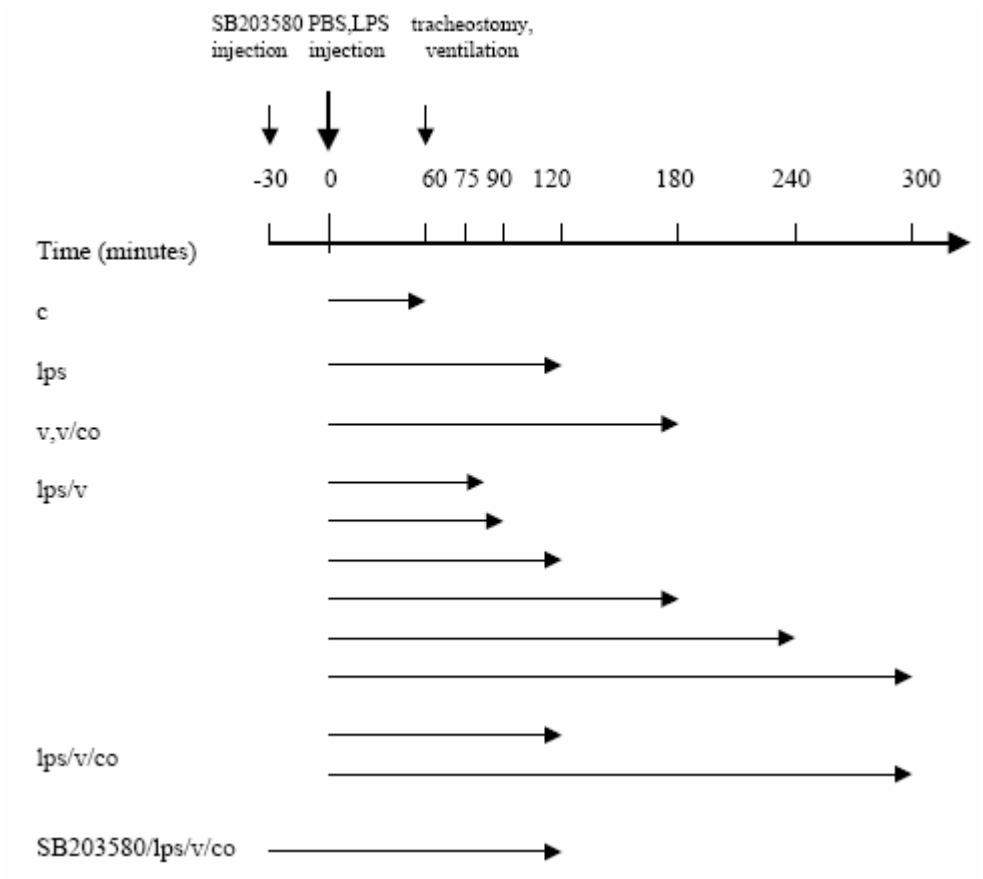


Figure legend:

Control (c) (n=11) animals were PBS treated, after 1hour tracheostomy was performed and animals were sacrificed immediately.

LPS (lps) (n=9) animals were LPS treated, after 1 hour tracheostomy was performed, and animals were sacrificed 1 hour after tracheostomy.

Ventilation (v) (n=9) animals were injected with PBS. After 1 hour tracheostomy was performed, and animals received 2 hours mechanical ventilation.

Ventilation/CO (v/co). (n=9) animals were PBS treated. Tracheostomy was followed by 2 hours of mechanical ventilation with 250ppm CO mixed with room air.

LPS/ventilation (lps/v) (n=24) animals were LPS treated, and mechanically ventilation following tracheostomy for 6 different timepoints. Three animals were ventilated for 15 minutes, 30 minutes, 2hours, 3 hours and 4 hours. Nine animals were ventilated for 1 hour.

LPS/ventilation/CO (lps/v/co) (n=18), following LPS treatment and tracheostomy the animals were exposed to different concentrations of CO through the rodent ventilator for 1 or 4 hours. Nine animals were ventilated for 1 hour with 250 ppm CO. Three animals for 1 hour with 100ppm. Three animals for 1 hour with 10ppm CO. Three animals were ventilated for 4 hours with 250ppm.

SB203580/LPS/ventilation/CO (SB203580/lps/v/co) (n=8), animals were injected with SB203580 30 minutes prior to LPS treatment. Following tracheostomy, animals were ventilated with 250 ppm CO for 1 hour.

Figure 8. Schematic overview of cRNA target preparation, bioarray hybridization and detection in microarray (modified after AmershanBiosciences catalogue: Manual Target preparation and cDNA detection manual)

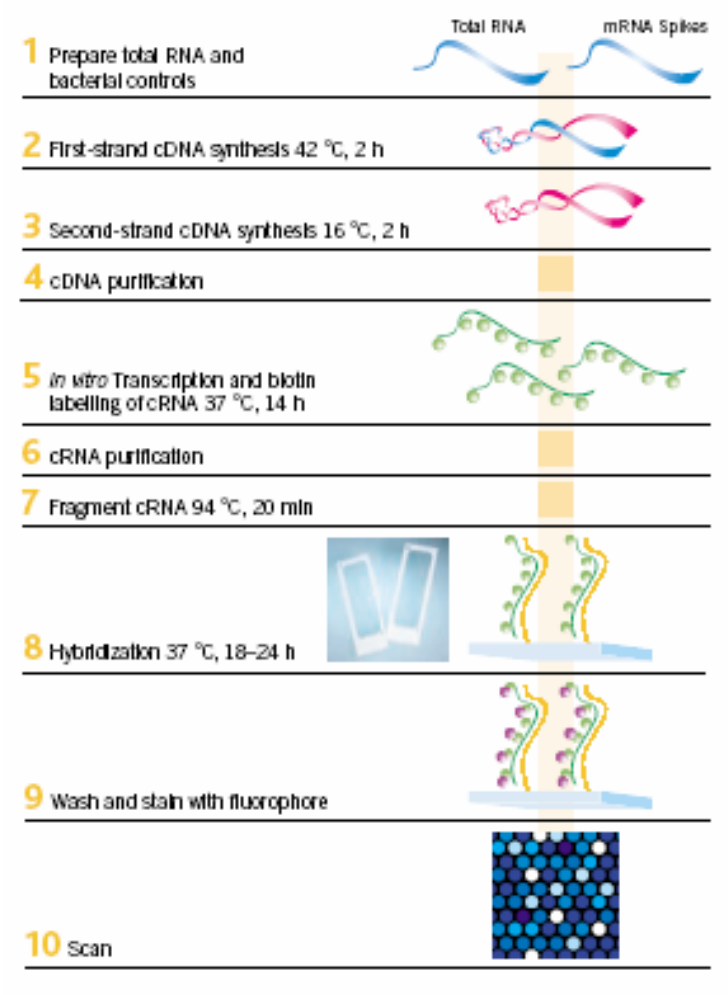
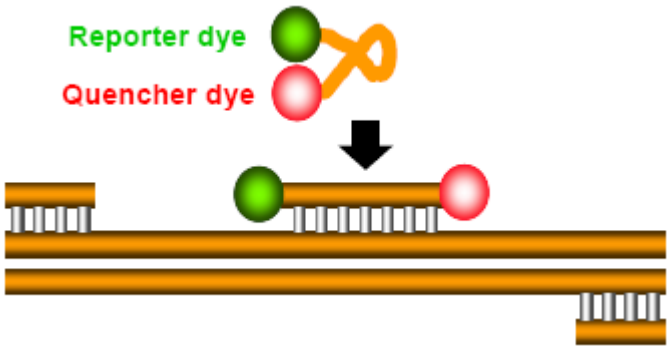
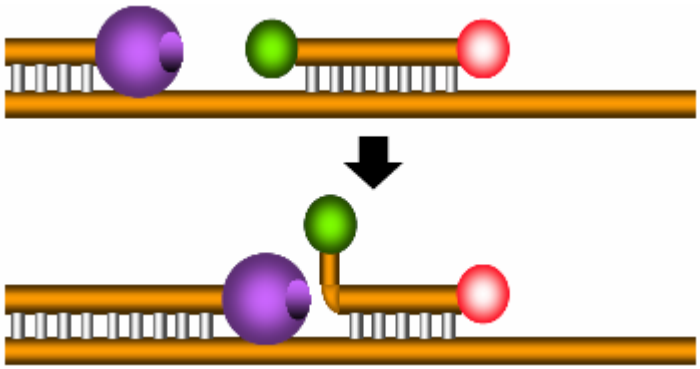


Figure 9. 5`nuclease assay with FRET (modified after Applied Biosystems catalogue: Real-time PCR vs. Traditional PCR)
A 5`nuclease assay



B Polymerase collides with TaqMan probe



C Cleavage of the TaqMan probe

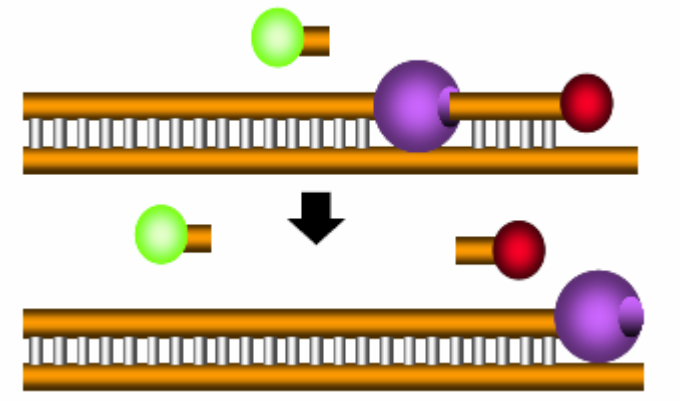
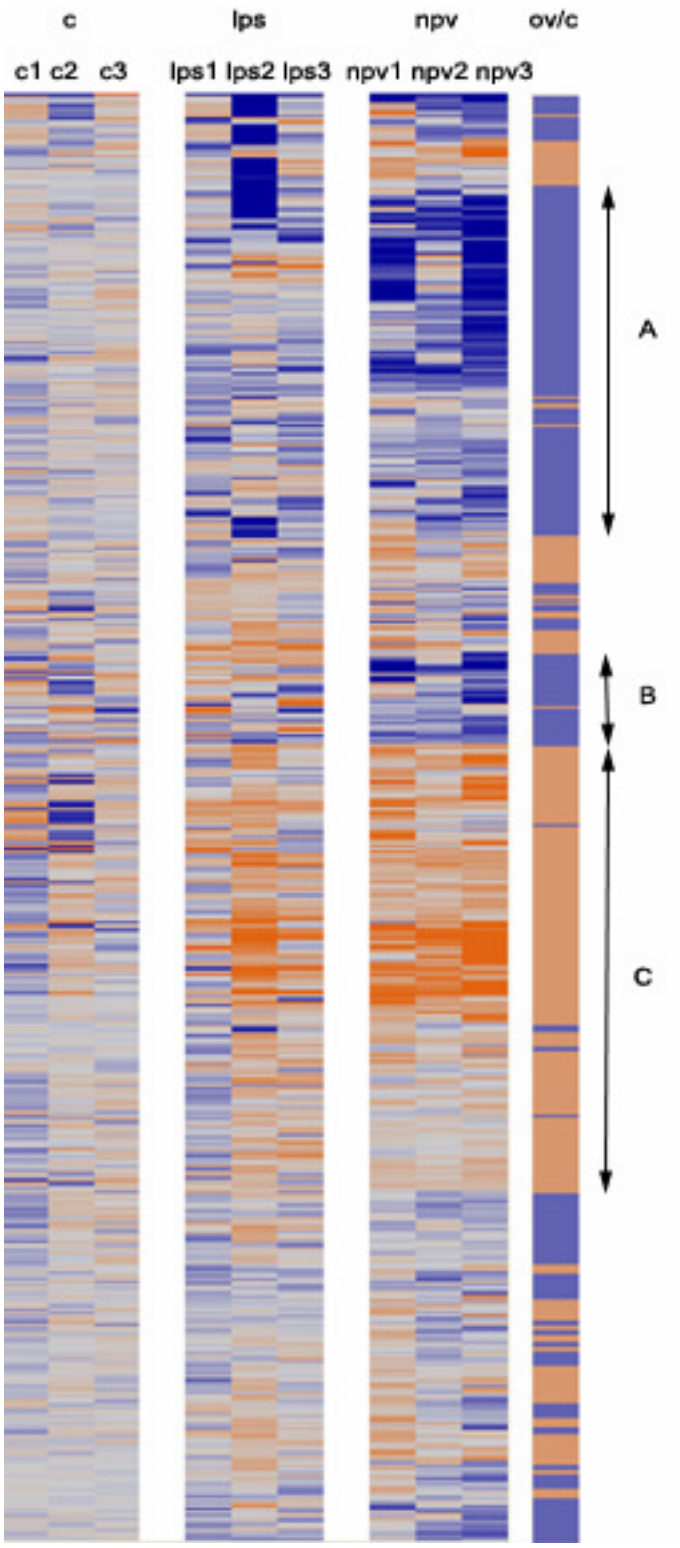
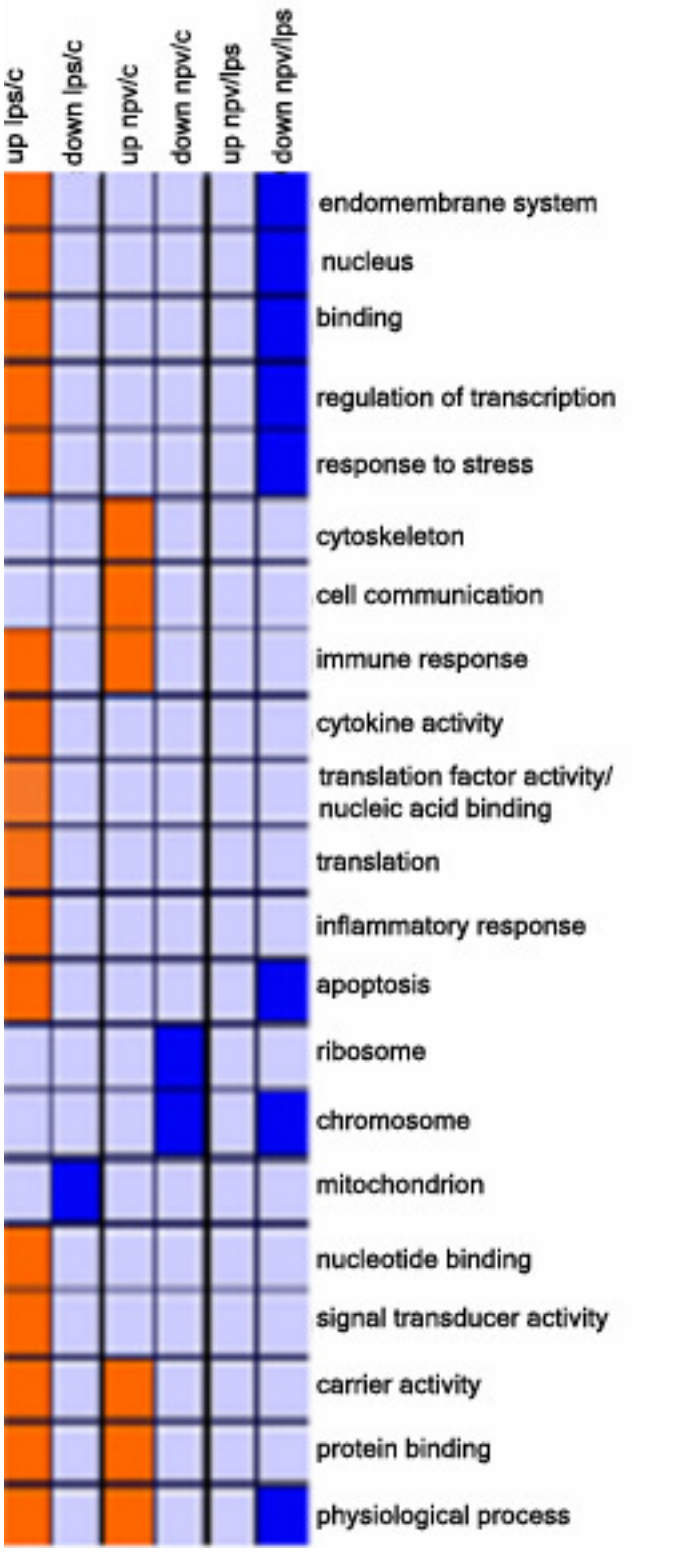


Figure 10. Hierarchical clustering of 5612 valid genes in mouse ventilator-induced lung injury



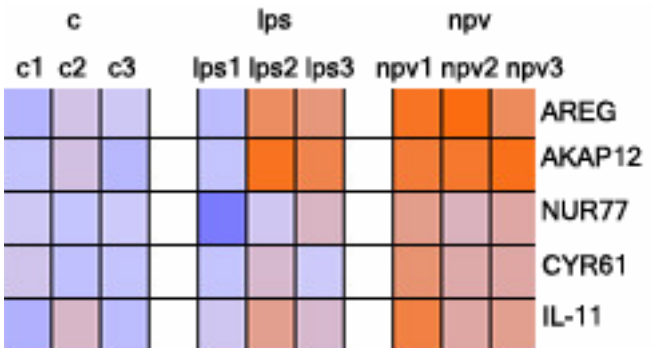
Legend: Each narrow column represents one animal. The animals treated in the same fashion are grouped in bigger columns and marked control (c), E. coli lipopolysaccharide treatment (lps) and overventilation (ov). N=3 animals/groups. Every row represents one gene. A gene is colored orange if its expression was increased. It is colored blue if the gene expression was decreased. Genes in gray did not show changes in expression. The last bar on the right shows the direction of gene expression changes (up or down) following ventilation expressed in an ov/c ratio format. Cluster A, B and C represents genes that behaved similar following one treatment but displayed different patterns when the 3 treatment groups were compared.

Figure 11. Functional grouping of 5612 valid genes in mouse ventilator-induced lung injury



Legend: Gene expression patterns are shown in $\log_2$ base ratios of treatment groups: lps/c, ov/c and ov/lps respectively. If the ratio is greater than 0 the small square is orange and if less than 0, it is blue. The square is colored gray if there was no significant change in the ratio of expression. Every row represents an important cellular function. Statistical significance was determined using hypergeometric model and corrected for multiple testing using FDR methods ($p<0.05$).

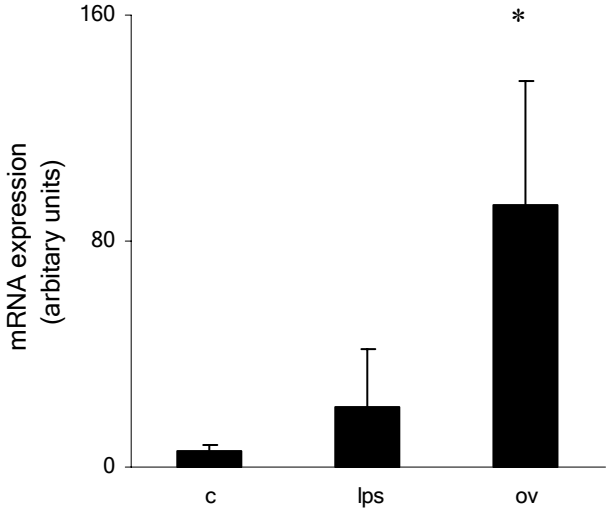
Figure 12. Gene expression profiles of 5 target genes in mouse ventilator-induced lung injury.



Legend: Each square represents an individual animals treated according to control, LPS or overventilation treatment regiment. AREG= amphiregulin, AKAP12= A protein anchor protein, NUR77= nuclear factor subfamily 4, group A, member 1, CYR 61= cystein-rich protein-61, IL-11= interleukin-11.

Figure 13. mRNA expression by RT-PCR.

A



Legend: (A) amphiregulin (AREG) (B) A protein anchor protein (AKAP12) (C) Nuclear factor subfamily 4, group A, member 1 (NUR77), (D) cystein-rich protein-61 (CYR61), (E) interleukin-11 (IL-11) expression was significantly increased following overventilation when compared to control. Values represent mean ±SD. * represents statistical significance of OV versus C, $p<0.05$.

B

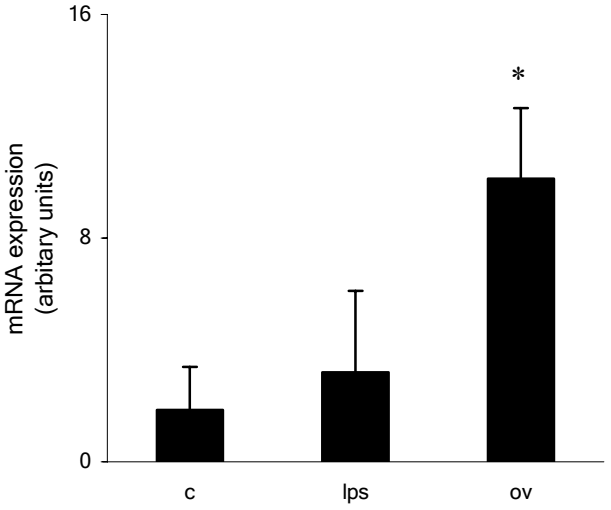
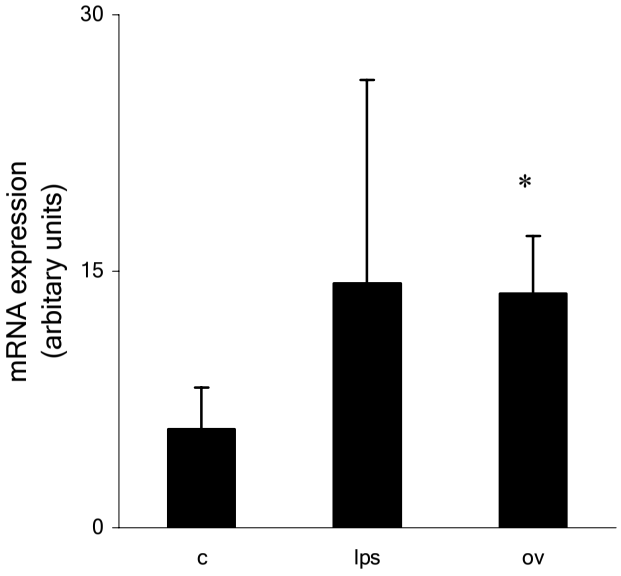


Figure 13.

C


30
15
0
mRNA expression
(arbitary units)
*
c
lps
ov

D

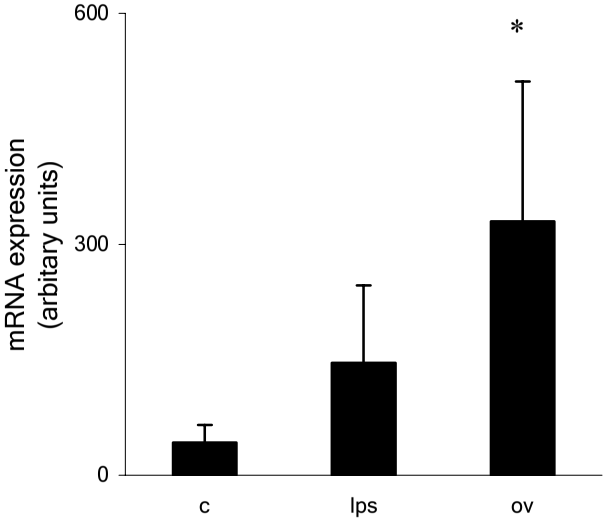

600
300
0
mRNA expression
(arbitary units)
*
c
lps
ov

Figure 13.
E

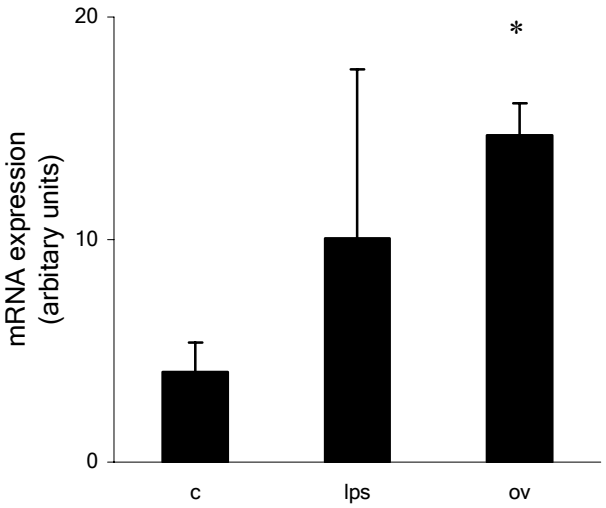
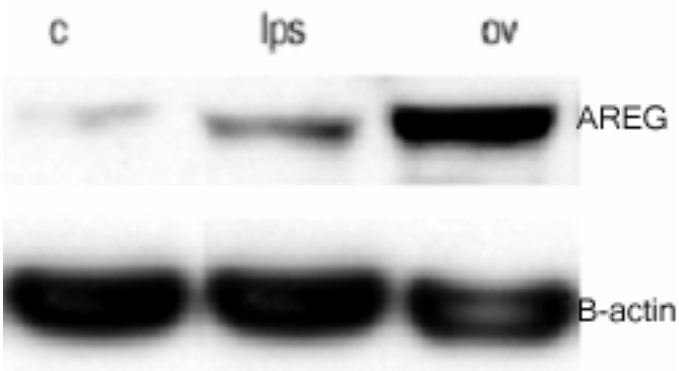
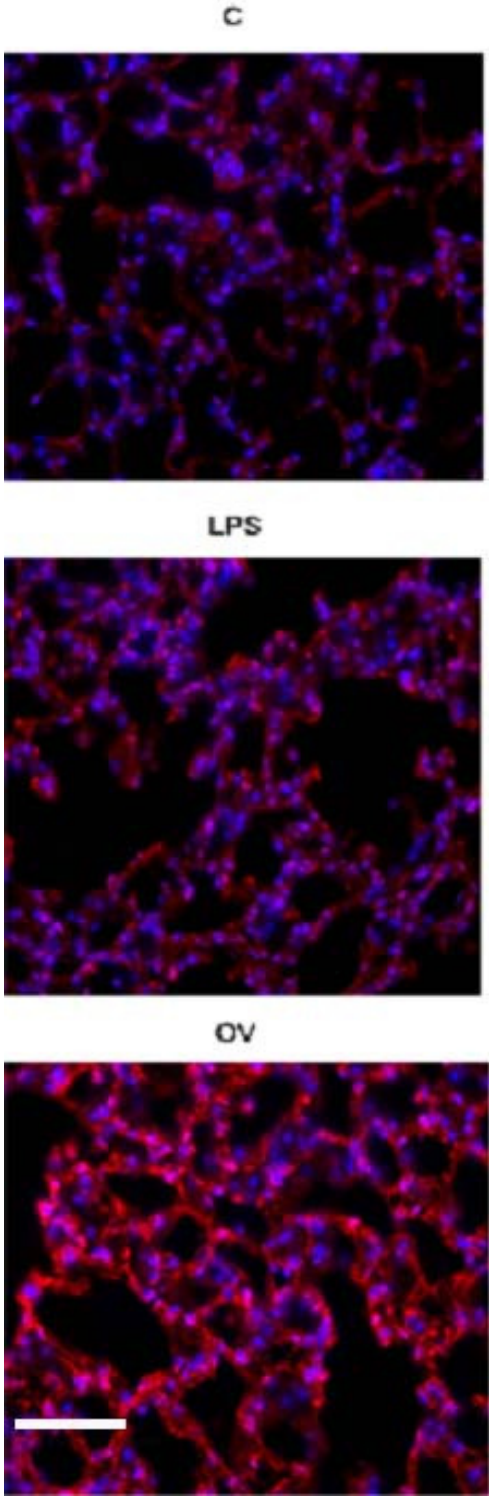


Figure 14. Amphiregulin protein expressions.



Legend: Amphiregulin protein expression increased following overventilation when compared to LPS and control treatments.

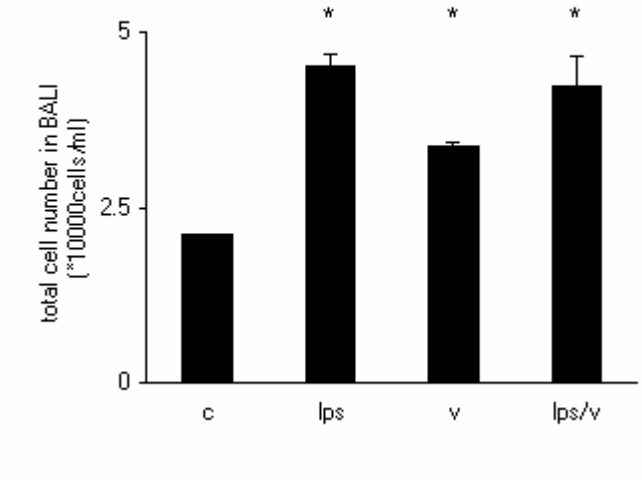
Figure 15. Immunhistochemical-staining of lung tissue



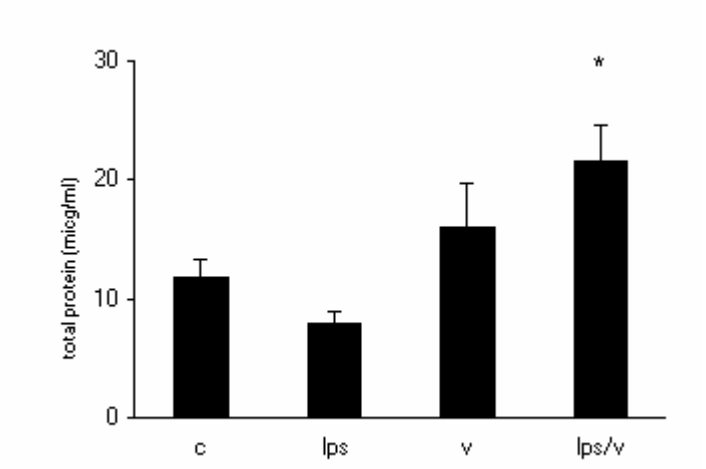
Legend: Lung sections were stained for amphiregulin. Red stain: immunofluorescent-labeled amphiregulin antibody. Blue stain: nuclear staining. Scale bar= 50 μm. Overventilation led to increased staining when compared to LPS and control treatments.

Figure 16. Effects of ventilation on BALF cell number and protein.

A

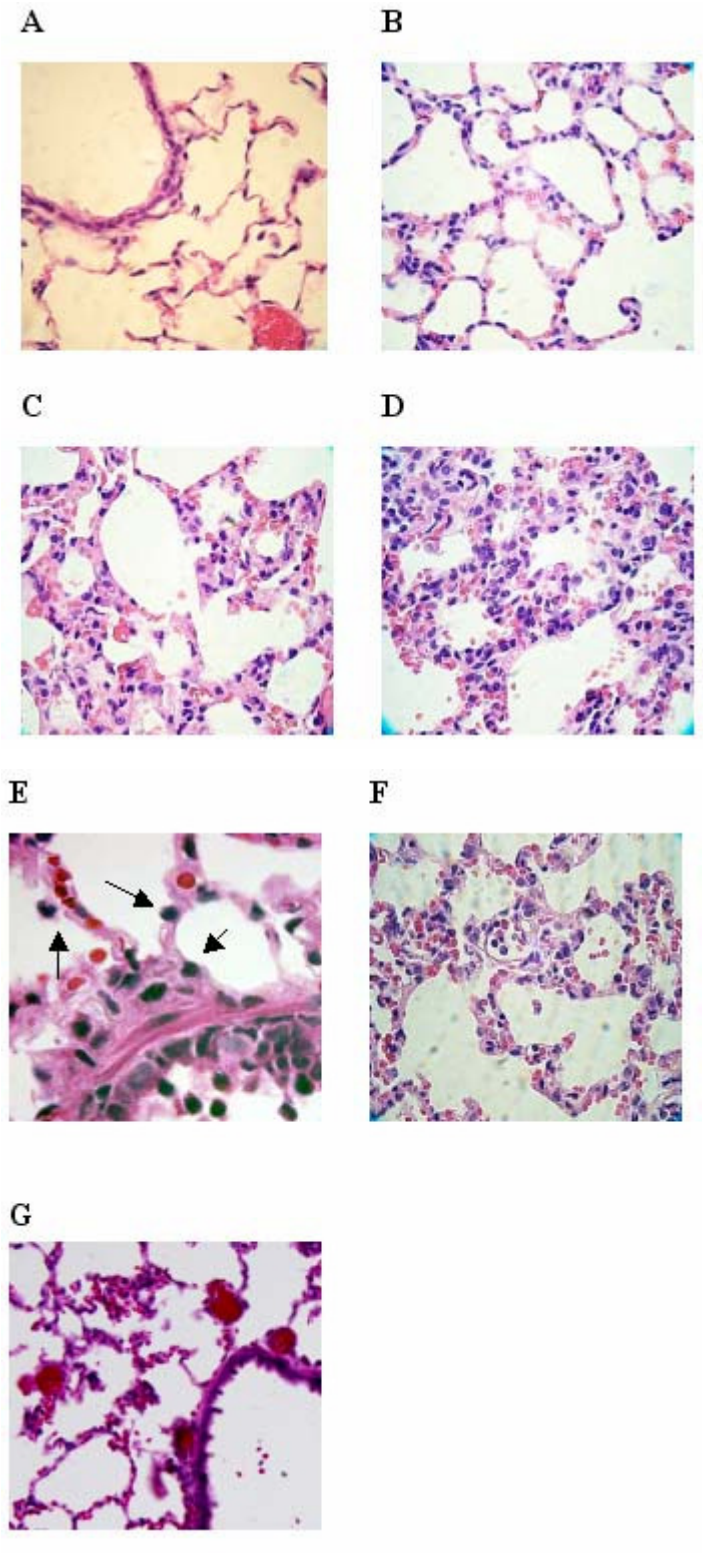


B



Legend: c= control animals were injected with PBS. After 1 hour tracheostomy was performed, and the animals were sacrificed immediately. lps= LPS animals were LPS treated, after 1 hour tracheostomy was performed, and the animals were sacrificed 1 hour after tracheostomy, v= ventilation animals were injected with PBS. After one hour tracheostomy was performed, and animals received 2 hours mechanical ventilation. lps/v=LPS/ventilation animals were LPS treated, after 1 hour tracheostomy was performed, and animals were mechanically ventilated for 1 hour. Values represent mean±SD. N=6 animals were used/ condition. * represents significant difference lps ($p<0.02$), v ($p< 0.029$) and lps/v ($p<0.025$) versus c.

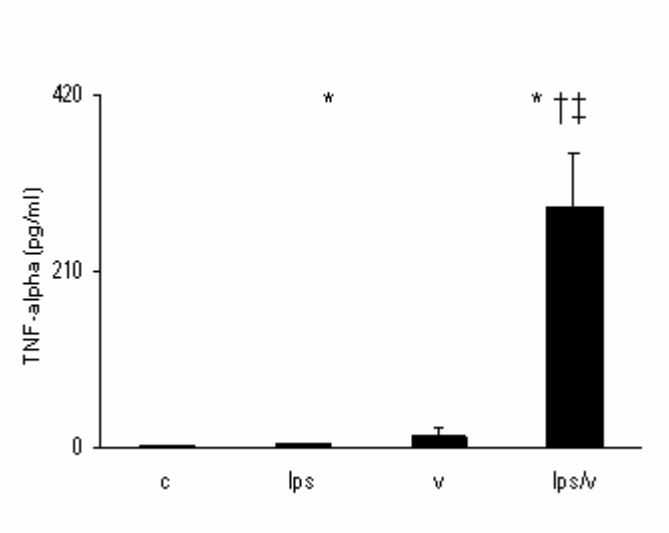
Figure 17. Lung tissues were stained with hematoxylin-eosin to demonstrate the magnitude of lung injury (x 40 magnification power)



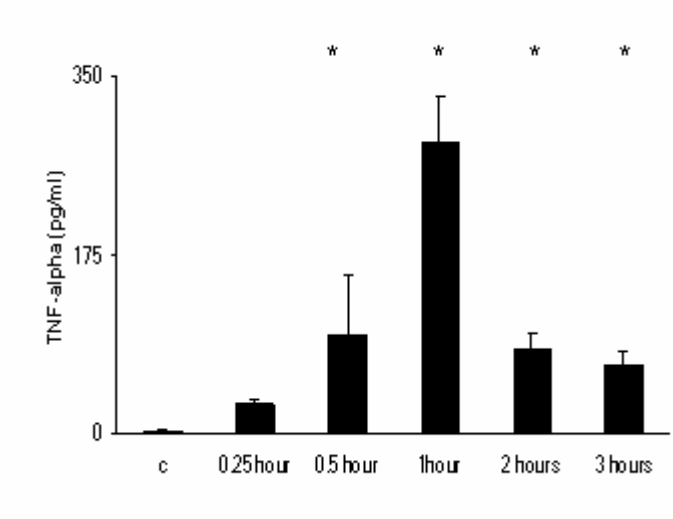
Legend: (A) Control. The alveoli and the capillaries are intact with no edema formation. (B) LPS treatment, the alveolar septi are thickened. Inflammatory cells are visible in the septi. The epithelial barrier is intact. (C) Ventilation, the alveolar septi are further thickened, partly ruptured, the inflammatory cell involvement in the septi is more characteristic. Red blood cells and edema fluid are present in the alveoli. (D) LPS/ventilation, the alveolar walls are ruptured and tissue is invaded by inflammatory cells. Red blood cells and edema fluid are present in the alveoli. (E) LPS/ventilation, (x100 magnification power). The arrows point to mononuclear leukocytes in the alveoli. (F) LPS/ventilation/CO, CO treatment modestly reduced hypercellularity and inflammation in the tissue when compared to LPS/ventilation. (G) SB203580/LPS/vent./CO treatment resulted in hemorrhage and inflammatory response in the tissue. lps/v/co= LPS/vent./CO animals were LPS treated, after 1hour tracheostomy was performed, and animals were mechanically ventilated with room air mixed with 250ppm CO for 1 hour. Three animals per condition were used to prepare histology and representative one is displayed.

Figure 18. Pro-inflammatory cytokine TNF-α levels (pg/ml) in the BALF

A

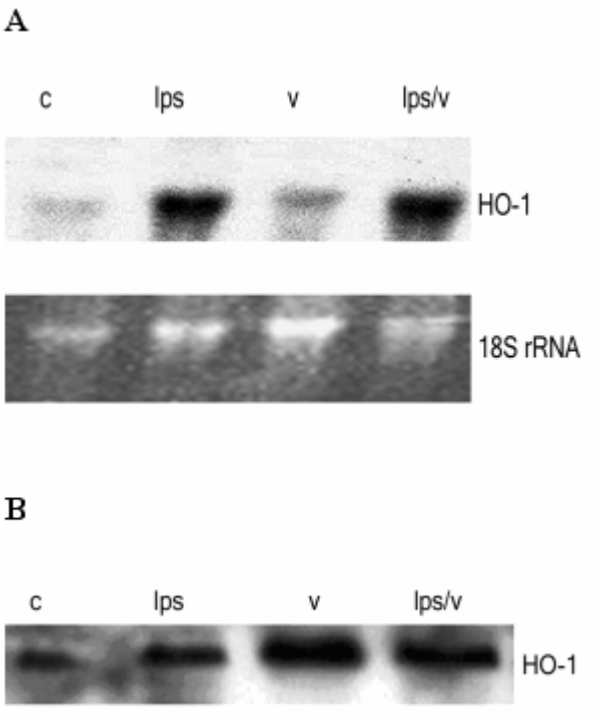


B



Legend: (A) LPS or LPS/ventilation treatment significantly increased TNF-α levels. Ventilation alone did not result in significant augmentation of the cytokine release. N= 6 animals were used per condition. * represents significant changes in TNF-α levels lps ($p<0.005$) and lps/v ($p<0.001$) versus c, † represents significant increase of TNF-α level in lps/v versus lps ($p<0.004$) and ‡ represents significant increase of TNF-α level lps/v versus v, $p<0.01$. (B) TNF-α levels (pg/ml) in LPS/ventilation condition. Animals received LPS injection, after 1hour tracheostomy was performed, and animals were ventilated for 15 minutes to 3 hours. After 30 minutes of mechanical ventilation, TNF-α level in the BALF was significantly higher in the treatment group than in control group. The cytokine level remained elevated in animals ventilated for 1, 2 and 3 hours. The peak TNF-α concentration was measured after 1-hour mechanical ventilation. C=control condition. N=3 animals per condition. * represents significant difference versus c ($p< 0.017$).

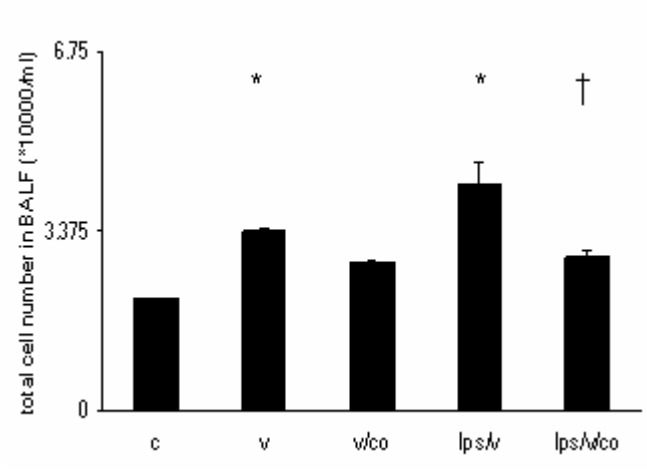
Figure 19. HO-1 mRNA and protein expression after ventilation.



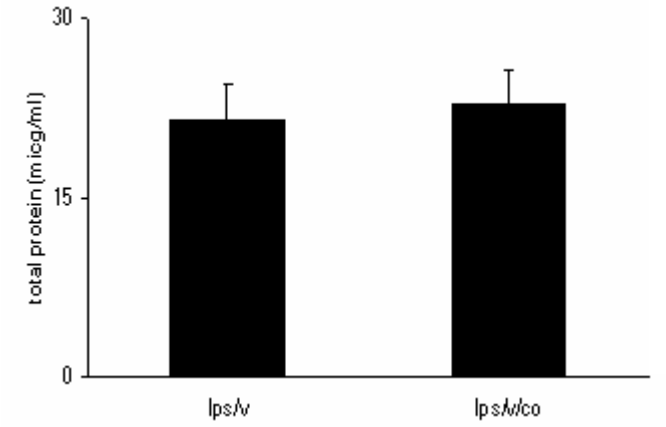
Legend: (A) Northern blot analysis of lung tissue shows increased HO-1 mRNA expression LPS, ventilation and LPS/ventilation treatment. 18S rRNA is included for RNA loading normalization. (B) LPS, ventilation and LPS/ventilation treatment increased the HO-1 protein expression in the lung tissue. Coommassie staining of gel was used for protein loading normalization (data not shown).

Figure 20. Effects of inhaled CO on indices of lung injury.

A



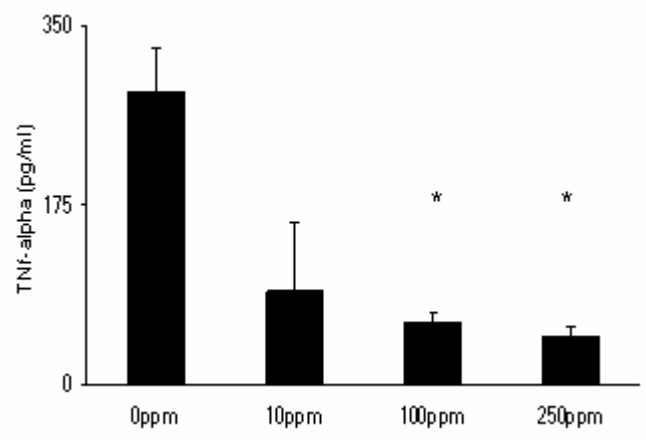
B



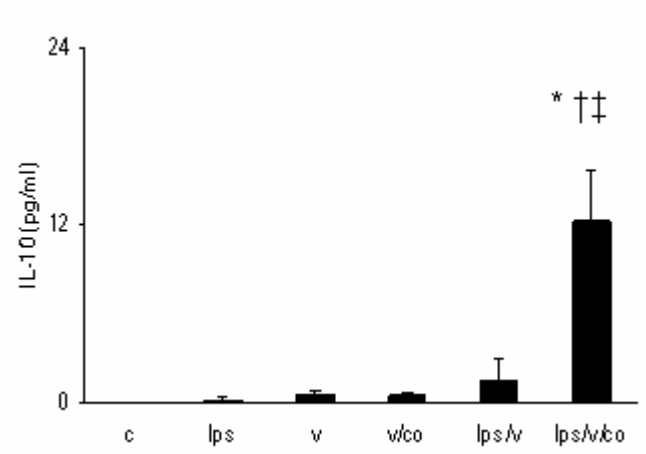
Legend: (A) BALF cell count; animals ventilated with 250ppm CO mixed with room air had less cells in BALF than their room air treated pairs. The difference was significant in LPS/ventilation/CO condition, in ventilation/CO condition a trend is visible but the difference it did not reach significance. The experiment time was 2 hours. N=6 animals were used/condition. * represents significant difference of v ($p<0.029$) and lps/v ($p<0.025$) versus c. † represents lps/v versus lps/v/co ($p<0.029$). v/co= ventilation/CO animals were PBS treated. After one hour tracheostomy was performed, and animals were mechanically ventilated for 2 hours with 250ppm CO mixed with room air. The same abbreviations will be used for the following figures. (B) Total protein measurement in the BALF (μg/ml). N=6 animals were used per condition.

Figure 20.

C



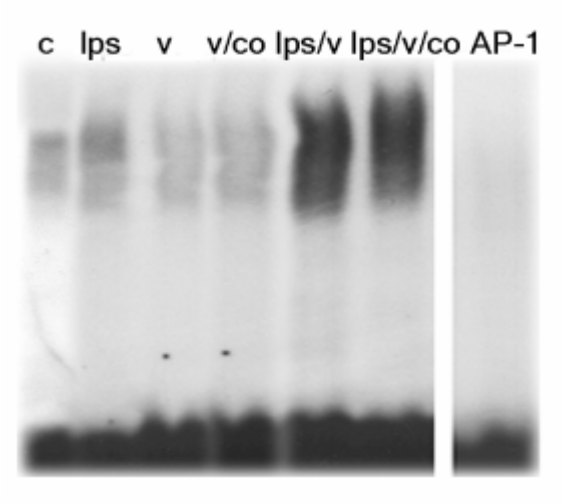
D



Legend: (C) TNF-α levels in the BALF after LPS/ventilation/CO treatment. Animals received LPS injection. After 1 hour tracheostomy was performed, and animals were ventilated with 10-250ppm CO mixed with room air for 1 hour. 100 and 250 ppm CO significantly reduced TNF-α levels in the BALF. 0ppm=LPS/ventilation condition. N=6 animals were used for 0 and 250ppm and n=3 animals were used for 10 and 100 ppm. * represents significant difference 100ppm ($p<0.024$) and 250ppm ($p<0.004$) versus 0 ppm. (D) IL-10 levels (pg/ml) in the BALF. LPS, ventilation and LPS/ventilation treatment did not affect IL-10 levels. 250 ppm CO treatment in the LPS/ventilation/CO condition significantly increased IL-10 level in BALF. When ventilation alone was combined with CO treatment (ventilation/CO condition) the effect was not observed. N=6 animals were used/condition. * represents significant difference of lps/v/co treatment versus c ($p<0.01$), † represents significant increase lps/v/co versus lps ($p<0.024$); ‡ represents significant increase lps/v/co versus lps/v ($p<0.022$).

Figure 21. Transcription factor AP-1 and NFκB activation was accessed by electrophoretic mobility shift assay (EMSA).

A



Legend: (A) EMSA showed activator complex-1 (AP-1) transcription factor activation after 2 hours of treatment. CO did not effect AP-1 activation. AP-1 = cold oligo, specific competition. (B) EMSA showed NF-κB transcription factor activation. LPS, ventilation and LPS/ventilation increased NF-κB activation. CO did not affect NFκB activation. NFκB= cold oligo, specific competition.

B

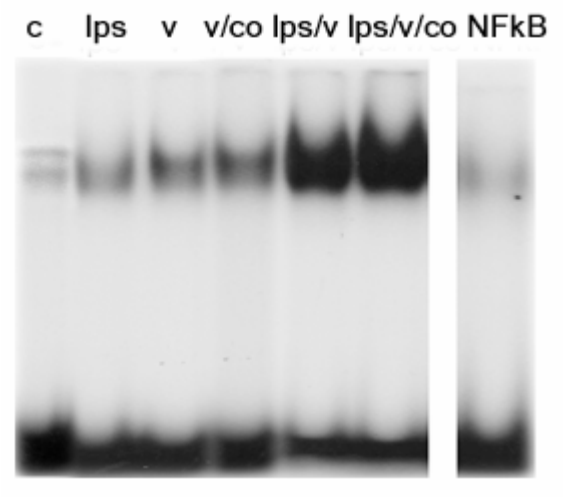
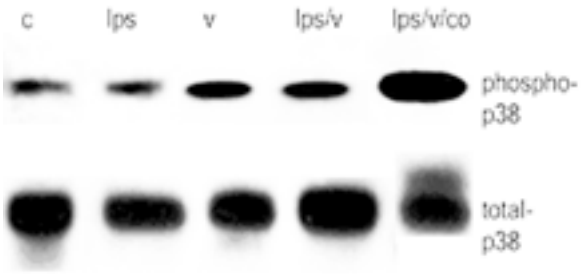
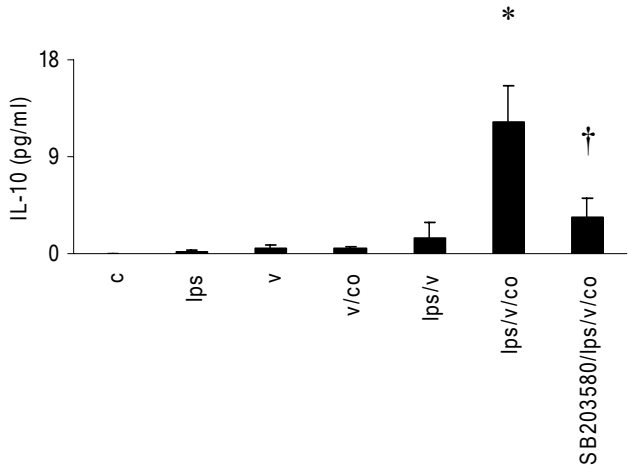


Figure 22. p38 mitogen-activated kinase (MAPK) activation in the lung tissue after CO treatment was detected by western blotting



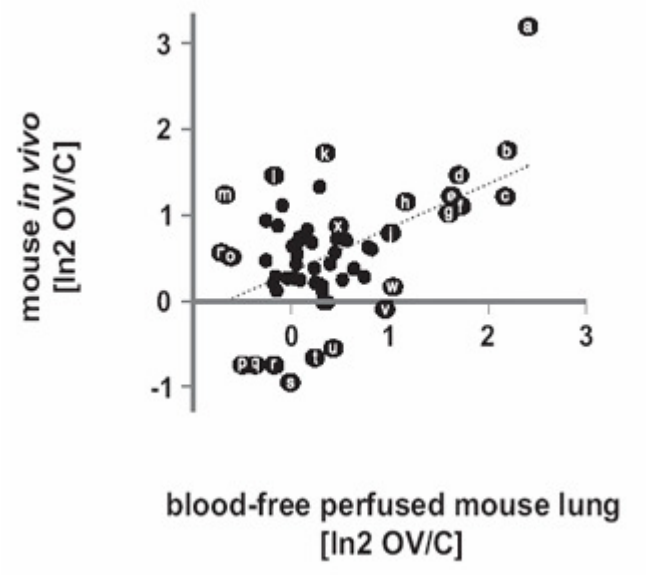
Legend: A specific phosphorylated p38 MAPK antibody was used. CO treatment (lps/v/co condition) significantly increased p38 MAPK protein expression. Total p38 MAPK is shown for protein loading control.

Figure 23. SB203580 p38 MAPK inhibitor attenuated IL-10 levels in the BALF



Legend: When animals were treated according to SB203580/LPS/ventilation/ CO condition IL-10 BALF levels were significantly lower than after LPS/ventilation/CO treatment. N=6 animals were used/condition. * represents significant increase in IL-10 levels lps/v/co versus c, ($p<0.01$). † represents significant difference between of lps/v/co versus SB203580lps/v/co ($p<0.038$).

Figure 24. Comparison of 62 genes from two microarray experiments.



Legend: Plotted on the X-axis are the data from the present study, on the Y-axis the data from Grigoryev et al (Reference#103). The correlation between both studies was Rho=0.302 (p=0.019, Sperman`s rank correlation coefficient). The dotted line represents the calculated regression line. a, Atf3, b, Areg; c,Ccl2; d,F3; e, Plaur; f,Il6; g,Gclc; h, Ptgs2; j, Arg2; k,Il1r2; l, Gabrd; m, Ifrd1; n,Aifl; o, Cxcr4; p,Admr; q, Ttf21; s,Npyr1; t,Cd79b; u, Mst1; v,Aqp1; w, Ywhaz: x. Il1b

Figure 25. Mayor cellular and intracellular events in acute lung injury (from reference#36)

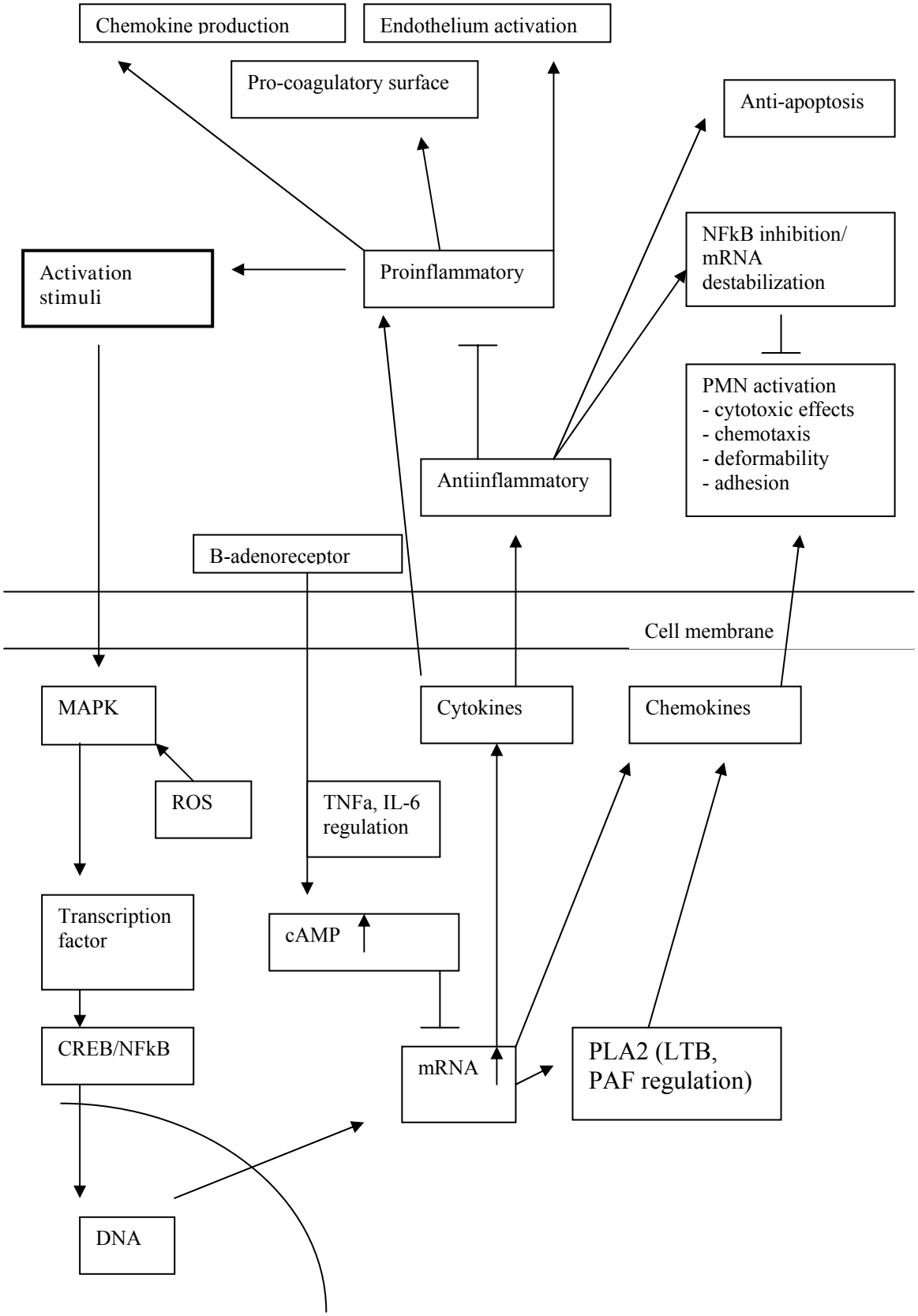


Figure 26. Transcriptional regulation required for neutrophil and endothelial activation in response to acute lung injury (from reference #42)

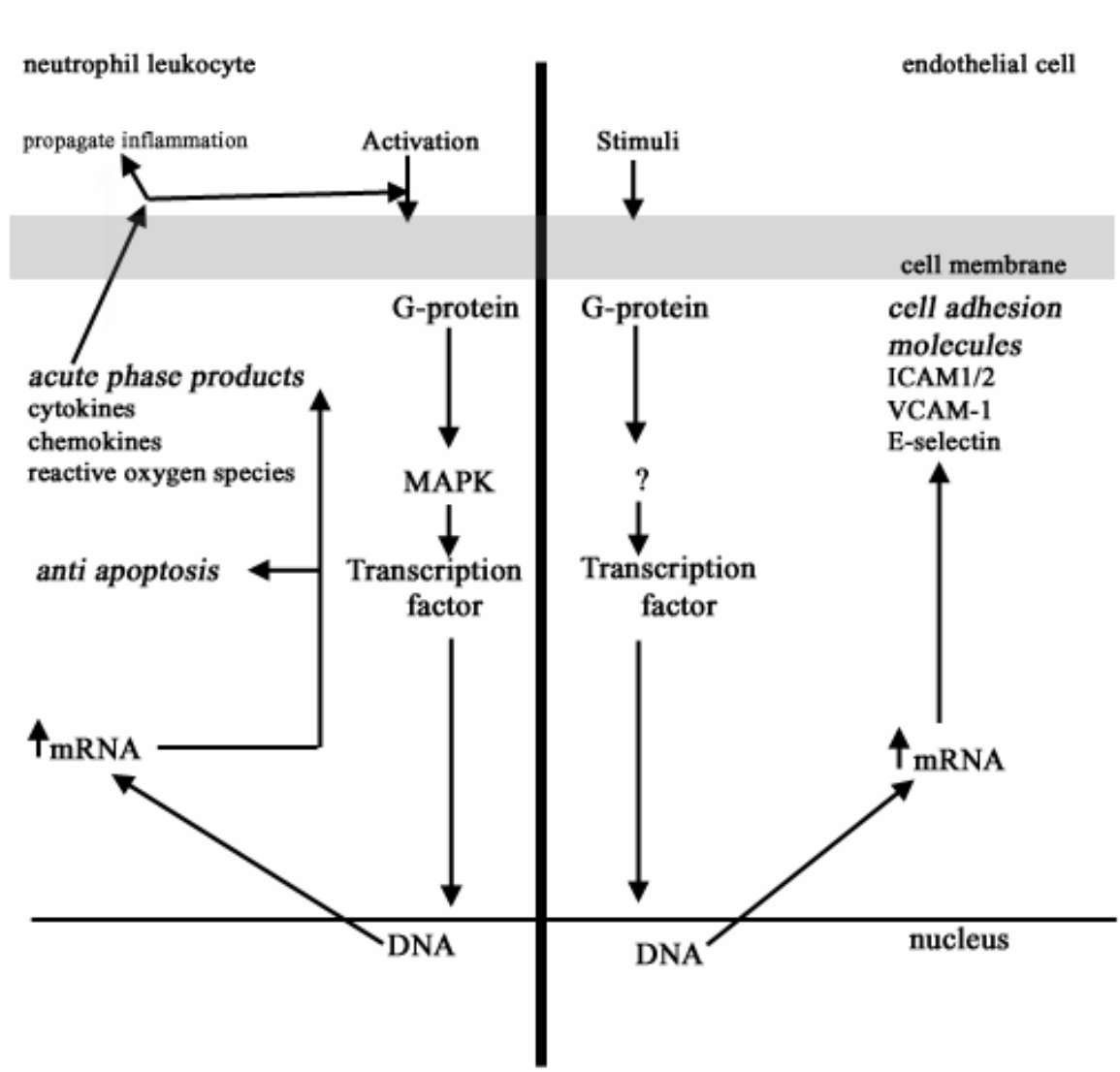
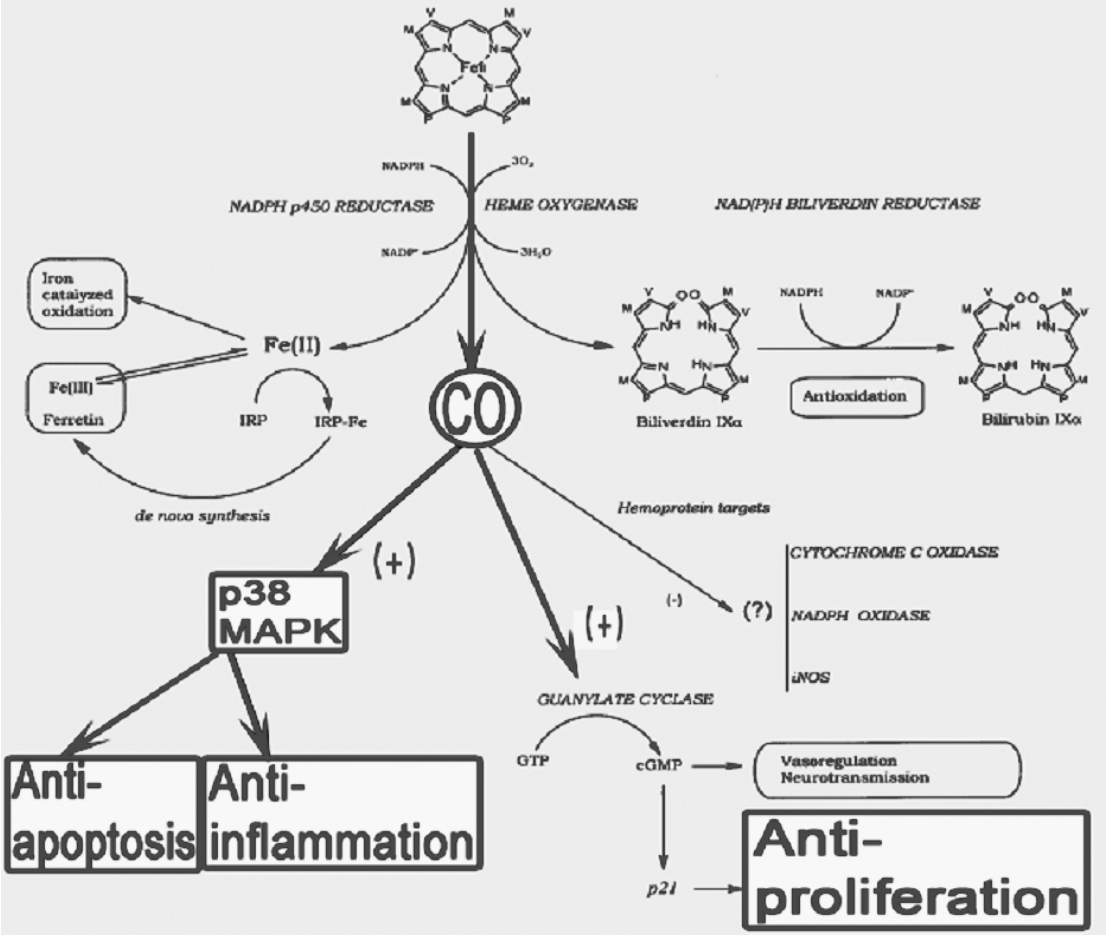


Figure 27 Heme degradation and the protective role of its metabolites (modified after Ryter S., Morse D., Choi A. Mol Cell Biochem 2002;234-235:249-263.)



11.Appendix

11.1 Inhaled carbon monoxide confers anti-inflammatory effects against ventilator-induced lung injury

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Supported by grants RO1 HL-55330 and HL-60234 from the National Institutes of Health, by the European Respiratory Society Short-Term Research Fellowship, by the Hungarian Respiratory Society Research Fellowship and by the Joseph Eötvös Public Foundation Scholarship.

This article has an online data supplement, which is accessible from this issue's table of content at www.atsjournals.org.

Abstract

Ventilator-induced lung injury is a major cause of morbidity and mortality in intensive care units. The stress inducible gene product, heme oxygenase-1 and carbon monoxide (CO), a major by-product of heme oxygenase catalysis of heme, have been shown to confer potent anti-inflammatory effects in models of tissue and cellular injury. In this study, we observed increased expression of heme oxygenase-1 mRNA and protein in a rat model of ventilator-induced lung injury. To assess the physiological function of heme oxygenase-1 induction in ventilator-induced lung injury, we determined whether low concentration of inhaled CO could serve to protect the lung against ventilator-induced lung injury. Low concentration of inhaled CO significantly reduced tumor necrosis factor-α levels and total cell count in lavage fluid, while simultaneously elevating levels of anti-inflammatory interleukin-10 levels. To better characterize the mechanism of CO-mediated anti-inflammatory effects, we examined key signaling pathways, which may mediate CO-induced anti-inflammatory effects. We demonstrate that inhaled CO exerts anti-inflammatory effects in ventilator-induced lung injury via the p38 mitogen-activated protein kinase pathway but independent of AP-1 and NF-κB pathways. Our data lead to a tempting speculation that inhaled CO might be useful in minimizing ventilator-induced lung injury.

Key words: p38 MAPK, cytokines, heme oxygenase-1

Introduction

According to an international study, an average of 39% of intensive care unit patients requires mechanical ventilation worldwide (1). Many of these patients develop ventilator-induced lung injury (VILI) (2). Eventually VILI contributes to acute respiratory distress syndrome (ARDS), which has a 40-50% mortality rate (3). While clinical trials showed that ARDS/VILI related mortality could be attenuated with lower tidal volume ventilation, positive end-expiratory pressure (PEEP) ventilation and more recently, with recruitment maneuver combined with protective ventilation strategy, the syndrome remains a major problem in intensive care units (3, 4, 5).

It is established that mechanical ventilators that apply high volumes and pressures can lead to increased alveolar-capillary permeability (6). This loss of compartmentalization results in increased fluid influx to the alveoli from the capillaries, causing pulmonary edema. The injured or ruptured cells attract neutrophil leukocytes and activate alveolar macrophages, causing inflammation in the lung (6). Tremblay and colleagues have suggested that ventilation can provoke an inflammatory response in the distal airway and alveolar cells (7), manifested by increased production of pro-inflammatory cytokines. It is speculated that the released pro-inflammatory cytokines could enter the circulation causing inflammation in other systemic organs. In addition, the previously diseased or injured lungs are more susceptible to subsequent mechanical ventilation, releasing more pro-inflammatory cytokines than healthy lungs, perhaps reflecting the cumulative effects of multiple injuries (8, 9). Lipopolysaccharide (LPS), acid aspiration and cecal ligation/perforation induced sepsis are commonly used models for approximating previous lung injury in ventilator-induced lung injury models (10-12).

Accumulating data exist in the literature supporting the paradigm that the stress inducible heme oxygenase-1 (HO-1) or its catalytic by-product, carbon monoxide (CO), can confer potent cytoprotective effects in various models of tissue and cellular injury (13-19). One mechanism by which HO-1 or CO mediate a cytoprotective effect is via its potent anti-inflammatory properties (13,14). Our laboratory has recently demonstrated that exogenous administration of low concentration of inhaled CO can markedly decrease lung inflammation and confer potent cytoprotection in various tissue injury models (15-19).

The primary goal of the present study was to test the hypothesis that inhaled CO can confer protective effects in an animal model of VILI. We used intravenous LPS injection and/or a relatively injurious ventilator setting to induce lung inflammation, in ventilated animals in the presence or absence of inhaled CO. A protective effect of CO was observed. We then describe the potential mechanism by which CO confers anti-inflammatory effects against VILI. Some of the results of these studies have been previously reported in the form of an abstract (20).

Methods

Animal Preparation

See details in online data supplement (Table 1E).

Adult 275-375 g male Sprague-Dawley rats (n=88) were purchased from Harlan (Indianapolis, IN). Rats were allowed to acclimate for 1 week with rodent chow and water ad libitum prior to experimentations. All animals were housed in accordance with guidelines from the American Association for Laboratory Animal

Care. The Animal Care and Use Committee of the University of Pittsburgh approved the protocols.

Animals received either 3mg/kg Escherichia coli bacterium lipopolysaccharide (LPS), Serotype O127: BO (Sigma, St. Louis, MO) in 0.25ml Phosphate Buffered Saline (PBS) or PBS alone, injected into the tail vein under ketamine (75mg/kg) and acepromazine (2.5mg/kg) intraperitoneal anesthesia (Sigma). After one hour of spontaneous breathing, tracheotomy was performed and a canula was inserted into the trachea. We designed seven experimental conditions: control, LPS, ventilation, ventilation/CO, LPS/ventilation, LPS/ventilation/CO and SB203580/LPS/ventilation/CO (n=6/condition). Pairs of CO treated and non-treated animals were formed and treated one after another. Animals treated according to condition ventilation, ventilation/CO, LPS/ventilation, LPS/ventilation/CO and SB203580/LPS/ventilation/CO received 26ml/kg tidal volume mechanical ventilation with room air or with 250 parts per million (ppm) CO mixed with room air for 15minutes to 4 hours without PEEP. Condition SB203580/LPS/ventilation/CO were injected intraperitoneally with SB203580 p38-kinase inhibitor (20mg/kg) 30 minutes prior to the experiment (21).

Arterial blood pressure and arterial blood gases were measured in condition LPS/ventilation and LPS/ventilation/CO (pressure transducer UFI, Morro Bay, CA; blood gas analyzer Radiometer ABL5, Copenhagen, Denmark).

CO dose-response experiment

LPS/ventilation/CO treated animals received 10, 100 or 250 ppm CO (n=3-6/dose). The concentration of CO was adjusted via a flow meter; the mixed gas runs to a chamber, which was connected to the rodent ventilator.

Cellular assays (ELISA, western and northern blots)

The cytokine analysis of BALF was carried out as described, using rat specific kits (R&D, Minneapolis, MN) (7). Total protein concentration was determined with Coomassie Plus 200 Protein Assay (Pierce, Rockford, IL). Western blot analysis was carried out as previously described for HO-1 and p38 MAPK phosphorylation (18). Total cellular RNA was extracted from lung tissue and Northern blot analysis was performed for HO-1 gene expression as previously described (17).

Electrophoretic mobility shift assay

Mobility shift assays were performed as described by Barberis (11) with minor modifications. The binding activity was determined after incubation of 4 μg of nuclear protein extract with either ^{32}P-labled 22-mer oligonucleotide encompassing the activator protein-1 (AP-1) binding site (5`-CTAGTGATGAGTCAGCCGCATC-3`, Promega, Medison, WI) or NF-κB binding site (5`-AGTTGAGGGGACTTTCCCAGGC-3`, Promega).

BALF total and differential cell count and tissue histology

See details in online data supplement.

Total cell number was counted with hemocytometer from resuspended cell pellet (Hausser, Horsham, PA). BALF and paraformaldehyde fixed lungs were stained with hematoxylin-eosin for qualitative cell count and histology. An experienced pathologist analyzed the lung tissues in a double-blind fashion.

Statistics

Results are presented mean± SD. Kruskal-Wallis test was performed for multiple group comparison and intergroup differences were analyzed with Wilcoxon Rank Sum Test (22) with SPSS statistics software (SPSS, Chicago, IL). Significance level was $p<0.05$.

Results

Ventilation enhances LPS-induced lung injury

To determine the magnitudes of lung injury caused by LPS, ventilation, and LPS/ventilation we performed two-hour experiments. LPS animals were sacrificed 2 hours after injection of LPS. In the LPS/ventilation condition, animals received LPS injection, and allowed to spontaneously breath for 1hour, and then treated with 1hour mechanical ventilation. Ventilation only animals received 2 hours of mechanical ventilation, and then sacrificed. For all animals, we measured total cell number and total protein from the BALF. LPS or ventilation alone significantly augmented the total cell number measured in the lavage fluid, as did LPS followed by ventilation (Figure 1A). LPS treatment followed by ventilation also significantly enhanced the total protein levels in BALF whereas ventilation or LPS alone did not increase BALF protein levels (Figure 1B). Figure 2 demonstrates the hematoxylin-eosin stained histology of the lung tissue following 2 hours treatments. When compared to PBS treatment (Figure 2A), LPS or ventilation alone caused inflammatory cells infiltration into the alveolar septi and thickening of the alveolar wall (Figure 2B,C). The combined effect of LPS and ventilation was the most injurious that resulted in the destruction of the alveolar structure (Figure 2D). Greater magnification shows

infiltrating mononuclear leucocytes into the alveoli (Figure 2E). The histology further supports the finding that ventilation further enhances LPS-induced lung injury.

Pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) levels dramatically increased in BALF after LPS/ventilation treatment compared with the control or ventilation alone treatment conditions (Figure 3A). Kinetic experiments show significantly elevated TNF-α levels in LPS/ventilation treated animals after 30 minutes ventilation. The maximal TNF-α level was measured after one-hour ventilation on the 3-hour ventilation time course (Figure 3B).

VILI induces HO-1 expression

In order to examine whether VILI can induce expression of the stress inducible HO-1, we performed Northern and Western blot analyses to determine HO-1 expression levels in the lung tissues after ventilation. As depicted in Figure 4, LPS or mechanical ventilation alone increased HO-1 gene and protein expression. The use of LPS, as a primer of lung injury, followed by ventilation increased HO-1 mRNA and protein expression the most when compared to controls (Figure 4A, B). These data suggest that HO-1, an important cellular stress response gene product, may play a role in defense against VILI.

Inhaled CO inhalation attenuates ventilator-induced lung injury

Inhaled CO significantly reduced the total cell number increased by LPS/ventilation in the BALF (Figure 5A). Ventilation alone with CO (ventilation/CO condition) markedly reduced the total cell number, but it did not reach significance. CO did not affect cell count in control and LPS treatment conditions (data is not shown). CO treatment did not affect the elevated total protein levels (Figure 5B).

We observed a dose-dependent decrease of the pro-inflammatory cytokine TNF-α in BALF when the animals inhaled CO during mechanical ventilation (Figure 5 C). We also measured the anti-inflammatory cytokine interleukin-10 (IL-10) in the BALF. LPS, ventilation or their combination did not affect IL-10 levels. CO treatment in LPS/ventilation/CO condition significantly increased IL-10 in the BALF. The effect was not observed in ventilation/CO condition (Figure 5D). CO did not have an effect on cytokine levels in control and LPS treatment conditions (data not shown). Modest decrease in hypercellularity and inflammation was observed in tissue histology after CO treatment (Figure 2F).

Differential cell count showed significantly reduced number of macrophages in the BALF after 2 hours treatment with 250ppm CO mixed with room air (Table 1). The number of neutrophil leukocytes in the BALF at this timepoint was negligible. To examine whether CO inhalation can effect neutrophil leukocyte infiltration to the alveoli, we performed LPS/ventilation and LPS/ventilation/CO condition experiments where animals received LPS injection, and allowed to spontaneously breath for 1hour, and then they were mechanically ventilated for 4 hours. Treatment with 250 ppm CO resulted in significantly reduced total cell count and neutrophil cell count in the BALF (Table 1). This finding suggests that CO may also reduce lung injury via inhibiting neutrophil leukocyte infiltration into the alveolar space.

Inhaled CO exerts negligible effects on hemodynamics and oxygenation

In order to confirm that mechanical ventilation and low dose inhaled CO used for our studies did not exert untoward effects on hemodynamics and gas exchange; we measured blood pressure and blood gases in LPS/ventilation and LPS/ventilation/CO conditions. Following tracheostomy a canula was inserted in the right carotid artery

and blood pressure was continuously measured during the ventilation. Blood was sampled from the canula for blood gases in the beginning and the end of mechanical ventilation. We did not observe statistically significant differences in blood pressure, pH, PCO_2, and PO_2 in LPS/ventilation and LPS/ventilation/CO conditions during the course of the experiment. Inter-group differences were not significant either. LPS treatment or ventilation alone had no effect on the hemodynamic of the animal model (data not shown). The carboxy-hemoglobin level was significantly elevated in CO treated animals, as expected (Table 2 and 3).

Mechanisms of CO-induced anti-inflammatory effects

Transcription factors are involved in cellular stress-induced gene expression and regulate biological processes including inflammation, cell proliferation and cell survival, all of which are important in conferring protection against cell and tissue injury (10). We examined whether transcription factors such as AP-1 and NF-κB are involved in CO-induced anti-inflammatory effects in VILI. LPS and ventilation both induced activation of AP-1 and NFκB (Figure 6A and B); however, CO treatment did not modulate the activation of AP-1 or NFκB binding activity compared with ventilation or LPS/ventilation treatment.

Another intracellular pathway that is activated in lung injury is p38 mitogen-activated protein kinase (MAPK) (23). In our model LPS and ventilation increased activation of p38 MAPK; CO significantly increased p38 MAPK activation when compared to ventilation and LPS (Figure 7). In order to examine whether CO-induced p38 activation exerted biological effects, we measured the levels of the anti-inflammatory molecule IL-10. We demonstrate that inhibition of p38 with the

chemical inhibitor SB203580 compound significantly attenuates CO induced IL-10 levels (Figure 8) and histology shows hemorrhagic, inflamed lung tissue (Figure 2G).

Discussion

Preclinical animal models of VILI have shown that the inflammatory milieu of pro- and anti-inflammatory cytokines plays a significant role in the pathology of VILI by causing biotrauma (7, 11, 22). It is speculated that cytokines released from the inflamed lungs to the blood may further cause deleterious physiologic effect on the host by injuring other organs, precipitating multiple organ failure (24).

We used a relatively injurious VILI model in rats, which resulted in lung injury that features inflammation, and edema as assessed by cell count, TNF-α production, and protein in the BALF (25, 26). We performed TUNEL assay in our lung tissues and did not observe evidence of cell death after VILI (data not shown). To better mimic a human disease course and maximize VILI, we used a sub lethal dose of LPS to prime and supplement ventilator induced lung injury, as often used by investigators (7, 9). Other injurious caustic agents such as hydrochloric acid or oleic acid were also applied to prime VILI in rodents (11, 27). Since it has been suggested that high volume ventilation may also affect hemodynamics (8), we measured blood gas and arterial blood pressure parameters and did not observe significant changes in blood pH, pCO_2, pO_2 and arterial blood pressure.

We initially observed a robust induction of HO-1 mRNA and protein in this model of VILI. Our laboratory and others have shown that HO-1 induction in response to cellular and tissue stress, *in vitro* or *in vivo*, is not only a reliable marker of cellular injury but also a physiologic response to defend against the inciting stress or cellular insult. Recently, our laboratory has provided evidence that CO, a major by-

product of heme catalysis by HO-1, mediates the protective effect of HO-1 (15-19). Thus, in view of our observation that HO-1 was markedly increased in VILI, we sought to assess whether CO could be responsible in mitigating VILI.

Using the same concentration of CO (250 ppm) we have used previously for *in vitro* and *in vivo* studies, we observed that CO could markedly attenuate the inflammatory responses of VILI. Inhaled CO significantly reduced the BALF cell count and TNF-α levels. Interestingly, we also observed that CO increased levels of the anti-inflammatory IL-10 in the BALF. These results correlate with the previous observations of Otterbein and colleagues in mice and murine macrophages (18).

Macrophages are the principal cell type found in the BALF after LPS/ventilation treatment. Macrophages are known to be sensitive to mechanical stress; thus, the changes in BALF total cell count might reflect an important role of macrophages in cytokine release in VILI (28). Belperio and colleagues described neutrophil leukocyte-predominant inflammatory response in mice following six hours high-volume (12ml/kg) mechanical ventilation (29). In our model neutrophil leukocyte infiltration to the alveolar space was observed after 4 hours of mechanical ventilation. Inhaled CO significantly reduced neutrophil recruitment to the alveoli. Additionally, inhaled CO also reduced BALF macrophage number at 2 hours. TNF-α is a well-known and well-investigated cytokine that has a pro-inflammatory effect in *in vivo* and *in vitro* models (10, 30) and IL-10 has anti-inflammatory activity in LPS-induced inflammation (31). Of note, it is interesting to note that CO did not affect the BALF protein levels suggesting that CO exerts negligible effects on pulmonary permeability. This observation provide us additional clue as to the differential and specific anti-inflammatory effect of CO which at this time appears to act as a

regulator of inflammation by attenuating pro-inflammatory cytokine and augmenting anti-inflammatory cytokine.

The signaling pathway by which CO acts as an anti-inflammatory agent is not fully understood. Although it is well established that CO activation of soluble guanylyl cyclase and cyclic-GMP (cGMP) mediates much of the vasodilatory effects (32), we did not observe a cGMP dependent effect in our VILI model (data not shown). Recent evidence suggests that pathways independent of cGMP are important in mediating CO signaling pathways. These cGMP independent pathways include the ERK MAPK in airway smooth muscle cell proliferation (33), p38-alpha MAPK and Egr-1 pathways in ischemia-reperfusion lung injury (34, 35), AP-1 in murine macrophages in response to LPS, and the p21 and p38 MAPK pathway in vascular smooth muscle cell proliferation (19, 36). We showed that NF-κB and AP-1 activation, two major pathways in ventilator-induced lung injury (7, 10), are not modulated by CO inhalation in VILI. The p38 MAPK is known to regulate TNF-α and IL-10 (37, 38) production. Although the molecular mechanism by which CO affects p38 MAPK to produce less TNF-α and more IL-10 needs to be further investigated, CO may have a posttranscriptional effect on TNF-α production (38).

We believe that our model can lead to a better understanding of the complex intracellular regulatory function of CO in lung injury. Based on the observations of this study, it is tempting to speculate that inhaled CO could represent a potential new therapeutic modality for counteracting VILI.

Acknowledgements

We thank W. Ameredes for their technical assistance and to T. Oury for the evaluation of histology.

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Figure legends

Figure 1. Effects of ventilation on BALF cell number and protein. (A) Total cell count showed significantly increased cell number after LPS, ventilation and LPS/ventilation treatment. (*10^4 cells/ml BALF). Values represent mean±SD in all figures. N=6 animals were used/ condition. * represents significant difference lps ($p<0.02$), v ($p< 0.029$) and lps/v ($p<0.025$) versus c. c= control animals were injected with PBS. After 1 hour trachesostomy was performed, and the animals were sacrificed immediately. lps= LPS animals were LPS treated, after 1 hour tracheostomy was performed, and the animals were sacrificed 1 hour after tracheostomy, v= ventilation animals were injected with PBS. After one hour tracheostomy was performed, and animals received 2 hours mechanical ventilation. lps/v=LPS/ventilation animals were LPS treated, after 1 hour tracheostomy was performed, and animals were mechanically ventilated for 1 hour. The same abbreviations are used for following figures. (B) Effects of ventilation on total protein measurement in the BALF supernatant (μg/ml). Only LPS/ventilation treatment increased significantly the level of total protein during the 2 hours period. N=6 animals were used/ condition. * represents significant difference versus c ($p<0.01$).

Figure 2. Lung tissues were stained with hematoxylin-eosin to demonstrate the magnitude of lung injury (x 40 magnification power). (A) Control. The alveoli and the capillaries are intact with no edema formation. (B) LPS treatment, the alveolar septi are thickened. Inflammatory cells are visible in the septi. The epithelial barrier is intact. (C) Ventilation, the alveolar septi are further thickened, partly ruptured, the inflammatory cell involvement in the septi is more characteristic. Red blood cells and edema fluid are present in the alveoli. (D) LPS/ventilation, the alveolar walls are

ruptured and tissue is invaded by inflammatory cells. Red blood cells and edema fluid are present in the alveoli. (E) LPS/ventilation, (x100 magnification power). The arrows point to mononuclear leukocytes in the alveoli. (F) LPS/ventilation/CO, CO treatment modestly reduced hypercellularity and inflammation in the tissue when compared to LPS/ventilation. (G) SB203580/LPS/ventilation/CO treatment resulted in hemorrhage and inflammatory response in the tissue. lps/v/co= LPS/ventilation/CO animals were LPS treated, after 1hour tracheostomy was performed, and animals were mechanically ventilated with room air mixed with 250ppm CO for 1 hour. SB203580/lps/v/co= SB203580/LPS/ventilation/CO animals were pretreated with SB203580. Following animals were LPS treated, after 1 hour tracheostomy was performed, and animals were mechanically ventilated with room air mixed with 250ppm CO for 1 hour. The same abbreviations are used for the following figures. Three animals per condition were used to prepare histology and representative one is displayed.

Figure 3. Pro-inflammatory cytokine TNF-α levels (pg/ml) in the BALF. (A) LPS or LPS/ventilation treatment significantly increased TNF-α levels. Ventilation alone did not result in significant augmentation of the cytokine release. N= 6 animals were used per condition. * represents significant changes in TNF-α levels lps ($p<0.005$) and lps/v ($p<0.001$) versus c, † represents significant increase of TNF-α level in lps/v versus lps ($p<0.004$) and ‡ represents significant increase of TNF-α level lps/v versus v, $p<0.01$. (B) TNF-α levels (pg/ml) in LPS/ventilation condition. Animals received LPS injection, after 1hour tracheostomy was performed, and animals were ventilated for 15 minutes to 3 hours. After 30 minutes of mechanical ventilation, TNF-α level in

the BALF was significantly higher in the treatment group than in control group. The cytokine level remained elevated in animals ventilated for 1, 2 and 3 hours. The peak TNF-α concentration was measured after 1-hour mechanical ventilation. C=control condition. N=3 animals per condition. * represents significant difference versus c ($p< 0.017$).

Figure 4. HO-1 mRNA and protein expression after ventilation. (A) Northern blot analysis of lung tissue shows increased HO-1 mRNA expression LPS, ventilation and LPS/ventilation treatment. 18S rRNA is included for RNA loading normalization. (B) LPS, ventilation and LPS/ventilation treatment increased the HO-1 protein expression in the lung tissue. Coommassie staining of gel was used for protein loading normalization (data not shown).

Figure 5. Effects of inhaled CO on indices of lung injury. (A) BALF cell count; animals ventilated with 250ppm CO mixed with room air had less cells in BALF than their room air treated pairs. The difference was significant in LPS/ventilation/CO condition, in ventilation/CO condition a trend is visible but the difference it did not reach significance. The experiment time was 2 hours. N=6 animals were used/condition. * represents significant difference of v ($p<0.029$) and lps/v ($p<0.025$) versus c. † represents lps/v versus lps/v/co ($p<0.029$). v/co= ventilation/CO animals were PBS treated. After one hour tracheostomy was performed, and animals were mechanically ventilated for 2 hours with 250ppm CO mixed with room air. The same abbreviations will be used for the following figures. (B) Total protein measurement in the BALF (μg/ml). N=6 animals were used/condition. (C) TNF-α levels in the BALF after LPS/ventilation/CO treatment. Animals received LPS injection. After 1

hour tracheostomy was performed, and animals were ventilated with 10-250ppm CO mixed with room air for 1 hour. 100 and 250 ppm CO significantly reduced TNF-α levels in the BALF. 0ppm=LPS/ventilation condition. N=6 animals were used for 0 and 250ppm and n=3 animals were used for 10 and 100 ppm. * represents significant difference 100ppm ($p<0.024$) and 250ppm ($p<0.004$) versus 0 ppm. (D) IL-10 levels (pg/ml) in the BALF. LPS, ventilation and LPS/ventilation treatment did not affect IL-10 levels. 250 ppm CO treatment in the LPS/ventilation/CO condition significantly increased IL-10 level in BALF. When ventilation alone was combined with CO treatment (ventilation/CO condition) the effect was not observed. N=6 animals were used/condition. * represents significant difference of lps/v/co treatment versus c ($p<0.01$), † represents significant increase lps/v/co versus lps ($p<0.024$); ‡ represents significant increase lps/v/co versus lps/v ($p<0.022$).

Figure 6. Transcription factor AP-1 and NFκB activation was accessed by electrophoretic mobility shift assay (EMSA). (A) EMSA showed activator complex-1 (AP-1) transcription factor activation after 2 hours of treatment. CO did not effect AP-1 activation. AP-1 = cold oligo, specific competition. (B) EMSA showed NF-κB transcription factor activation. LPS, ventilation and LPS/ventilation increased NF-κB activation. CO did not affect NFκB activation. NFκB= cold oligo, specific competition.

Figure7. p38 mitogen-activated kinase (MAPK) activation in the lung tissue after CO treatment was detected by western blotting. A specific phosphorylated p38 MAPK

antibody was used. CO treatment (lps/v/co condition) significantly increased p38 MAPK protein expression. Total p38 MAPK is shown for protein loading control.

Figure 8. SB203580 p38 MAPK inhibitor attenuated IL-10 levels in the BALF. When animals were treated according to SB203580/LPS/ventilation/CO condition IL-10 BALF levels were significantly lower than after LPS/ventilation/CO treatment. N=6 animals were used/condition. * represents significant increase in IL-10 levels lps/v/co versus c, ($p<0.01$). † represents significant difference between of lps/v/co versus SB203580lps/v/co ($p<0.038$).

Table 1 Total and differential cell count in BALF following LPS treatment and 2 or 4 hours of mechanical ventilation with and without carbon monoxide (CO)

	Total cell number ($*10^4$ cells/ml)	Macrophages ($*10^4$ cells/ml)	Neutrophil leukocytes ($*10^4$ cells/ml)
Control 2 hours	2.11± 0.5	2.08± 0.31	0±0
Control 4hours	2.34± 0.66	2.31± 0.33	0±0
LPS/ventilation 2 hours	4.235± 0.43	4.192±0.21 *	0±0
LPS/ventilation 4 hours	19.1± 1.8‡	16.32± 1.51‡	2.78± 0.27‡
LPS/ventilation/CO 2hours	2.9± 0.132	2.87± 0.06†	0±0
LPS/ventilation/CO 4 hours	13.75± 2.0§	13.51± 2.84	0.23± 0.17§

* p<0.03 LPS/ventilation 2 hours versus Control 2 hours condition, † p<0.029 LPS/ventilation/CO 2hours versus LPS/ventilation 2hours condition, ‡ p<0.01 LPS/ventilation 4 hours versus Control 4 hours condition , § p<0.05 LPS/ventilation/CO 4hours versus LPS/ventilation 4 hours condition, n=3 animals/condition

Table 2 Mean arterial blood pressure during mechanical ventilation with and without CO

Mean blood pressure (mmHg)	0 minutes	15 minutes	30 minutes	45 minutes	60 minutes
LPS/ ventilation*	82±5	80±3	100±5	107±7	118±6
LPS/ ventilation/ CO*	84±3	82±4	95±6	110±4	122±4

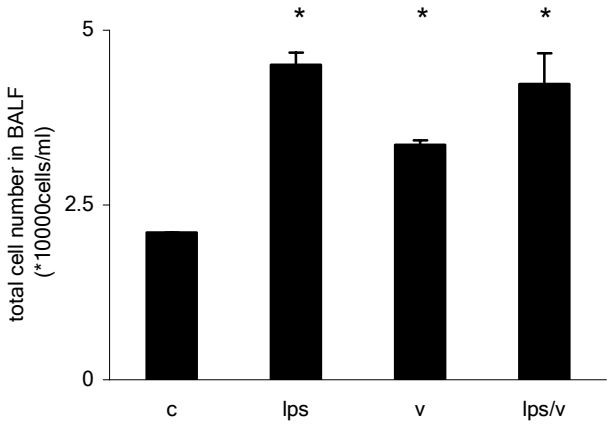
* n=3 animals/condition

Table 3 Blood gases and carboxy-hemoglobin (carboxy-Hbg) prior and after mechanical ventilation with and without CO

	pH		PCO_2 (mmHg)		PO_2 (mmHg)		carboxy-Hbg (%)	
	start	end	start	end	start	end	start	end
LPS / ventilation	7.34±0.06	7.22±0.05	22±5	40±3	104±10	86±12	6.5±1.2	6.2±0.8
LPS/ ventilation/ CO	7.32±0.02	7.26±0.03	23±4	46±5	108±18	90±19	5.8±1.7	14.5±2*

* $p<0.03$ versus LPS/ventilation treatment, n=3 animals/condition

Figure 1
A


total cell number in BALF
(*10000cells/ml)
5
2.5
0
*
*
*
c
lps
v
lps/v

B

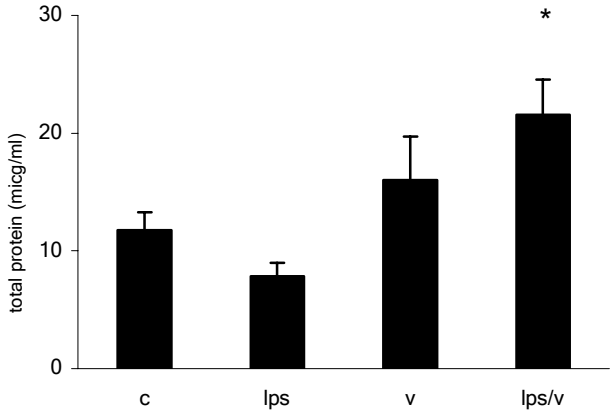
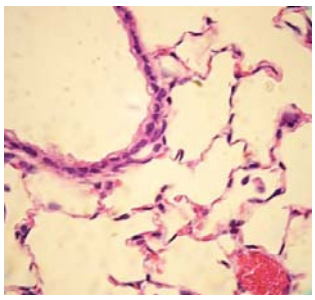

total protein (micg/ml)
30
20
10
0
*
c
lps
v
lps/v

Figure 2

A



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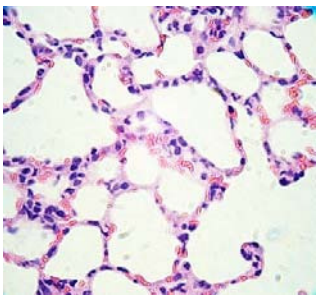

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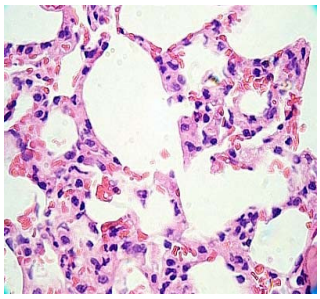

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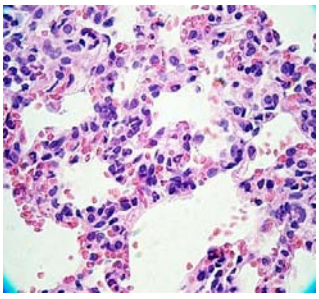

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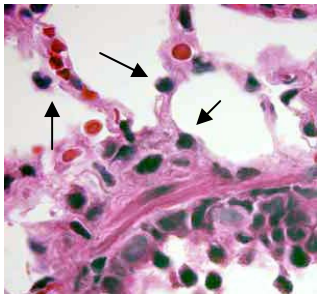

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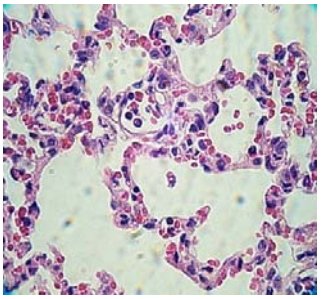

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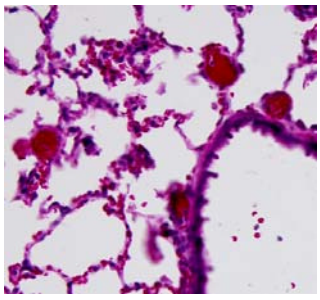

Figure 3
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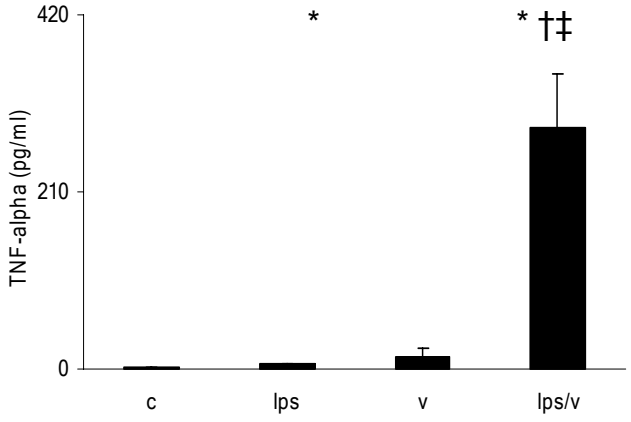

420
210
0
TNF-alpha (pg/ml)
*
* †‡
c
lps
v
lps/v

B

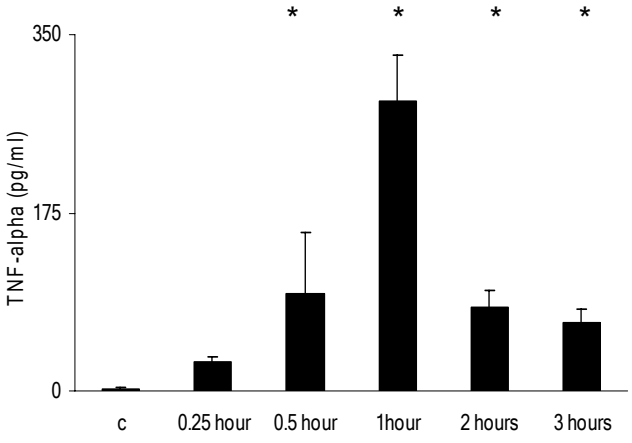
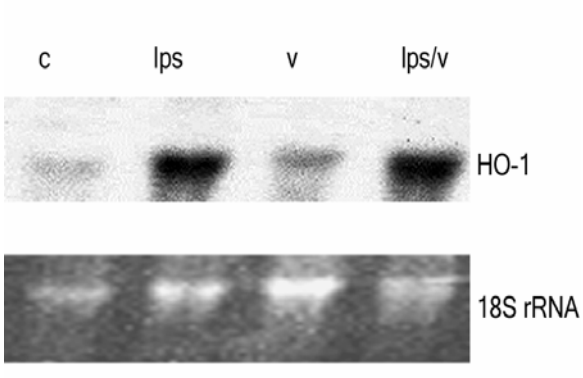

350
175
0
TNF-alpha (pg/ml)
*
*
*
*
c
0.25 hour
0.5 hour
1hour
2 hours
3 hours

Figure 4
A



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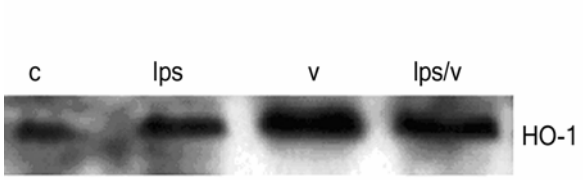
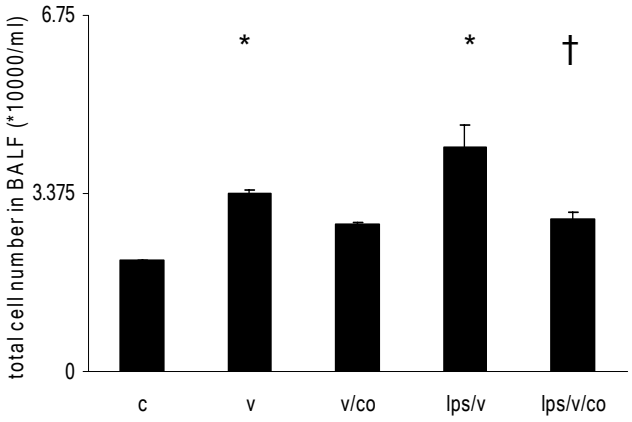
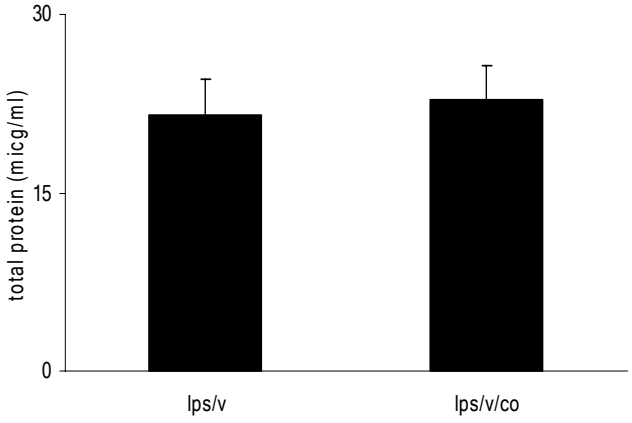


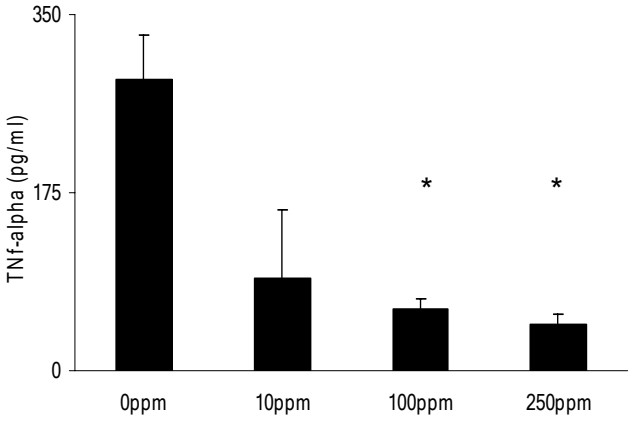
Figure 5
A


6.75
3.375
0
total cell number in BALF (*10000/ml)
*
*
†
c
v
v/co
lps/v
lps/v/co

B


30
15
0
total protein (micg/ml)
lps/v
lps/v/co

C


350
175
0
TNf-alpha (pg/ml)
*
*
0ppm
10ppm
100ppm
250ppm

D

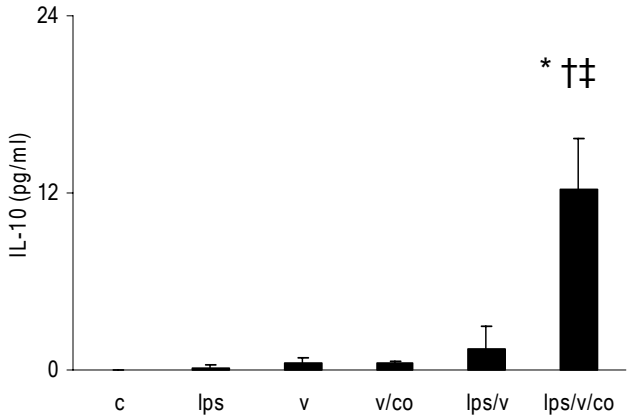
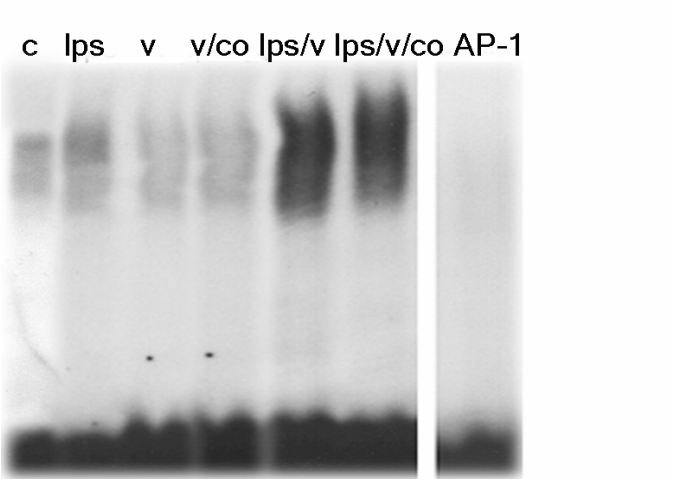

24
12
0
IL-10 (pg/ml)
* †‡
c
lps
v
v/co
lps/v
lps/v/co

Figure 6
A



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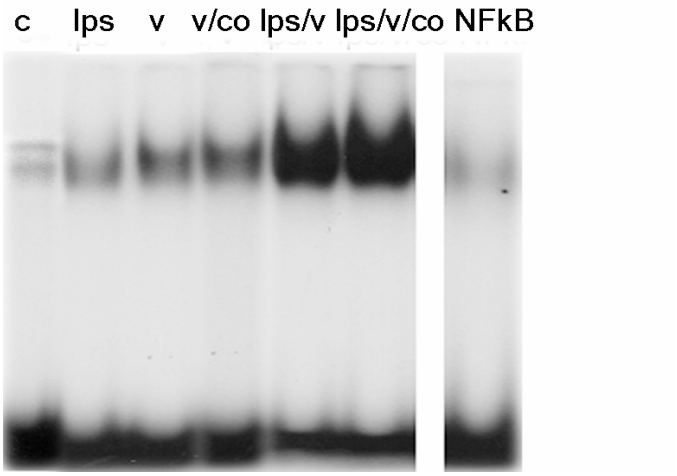


Figure 7

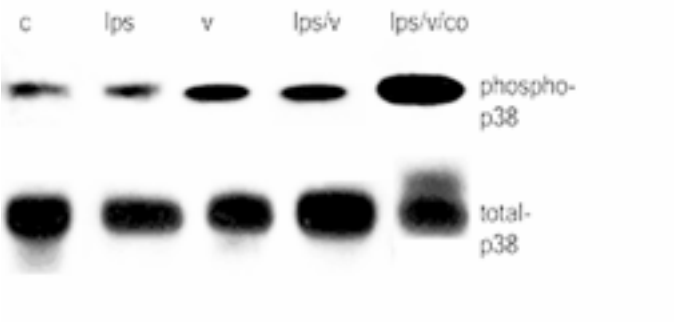
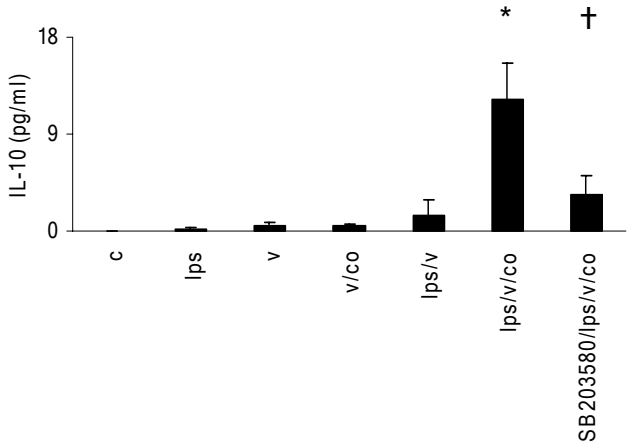


Figure 8



11.2 Gene expression profiling of target genes in ventilator-induced lung injury

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*Equal contributions from both laboratories.

Grants: R01-HL60234, R01-AI42365, R01-HL55330 awarded to A.M.K Choi; DFG Uh 88/4-2 to S. Uhlig. T. Dolinay is the recepient of a European Respiratory Society fellowship (Nr. STRF2003-012).

This article has an online data supplement, which is accessible from this issue's table of content at www.atsjournals.org.

Abstract

High-pressure mechanical ventilation is able to induce lung injury similar to that observed in acute respiratory distress syndrome. To gain a better understanding of the pathogenesis and mechanisms of ventilator-induced lung injury, we performed gene expression profiling analysis of 10500 mouse genes in isolated blood-free (to exclude genes from sequestered leukocytes) perfused mouse lungs exposed to low-pressure ventilation (10 cm H_2O), high-pressure ventilation (25 cm H_2O) or LPS treatment. LPS treatment and ventilation were found to co-regulate a large number of inflammatory and apoptotic genes. However certain growth factor-related genes, as well as genes related to general metabolism, cellular communication and the cytoskeleton, were only regulated by overventilation. The expression pattern of 5 genes (amphiregulin, gravin, NUR77, CYR61, interleukin-11) was confirmed with RT-PCR and the expression of amphiregulin also by immunohistochemistry and immunoblotting. These genes represent novel candidate genes in ventilator-induced lung injury.

Key words: overventilation, amphiregulin, microarray, ARDS, biotrauma

Introduction

Despite intensive investigation the mortality of acute respiratory distress syndrome (ARDS) remains high (1). Both patients at risk for ARDS and ARDS patients themselves (2,3) are prone to ventilator-induced lung injury (VILI) and benefit from low tidal volume ventilation (4). Clinical and animal studies suggest that the release of pro-inflammatory cytokines and other factors during alveolar overdistension contributes to lung injury (5-9). One important insight in this area is that ventilation with high distending pressures (overventilation) may activate, if by different signaling mechanisms, canonical inflammation pathways that become also activated by well-known pro-inflammatory stimuli such as bacterial endotoxin (9). For instance, both overventilation and LPS activate NF-κB (7,10) and MAPK (11), which subsequently activate chemokines, cytokines and adhesion molecules (9). An important question is to identify genes that are specifically activated by overventilation, but not by endotoxin. Such genes might provide specific targets to reduce the side effects of mechanical ventilation without interfering with the innate immune system. Microarray technology allows to study gene expression of a significant fraction of the genome and to identify candidate genes critical to a variety of lung diseases including chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, lung cancer and pulmonary hypertension (12-15). However, application of this approach to ventilator-induced gene expression *in vivo* is complicated by at least two factors: (i) in intact, non-injured animals alveolar overdistension is limited by both the chest wall and adjacent alveoli, (ii) infiltrating inflammatory cells may alter the gene expression profile. These two problems are circumvented in isolated blood-free perfused lungs. Therefore, in the present study we have used microarray analysis to study the effect of overventilation on gene

expression and compared this gene expression pattern to that induced by LPS. We identified 5 new candidate genes in the overventilation group and hypothesize that the gene products of Areg (amphiregulin), Akap12 (A protein anchor protein 12), Nur77 (nuclear receptor subfamily 4, group A, member 1), Cyr61 (cystein-rich protein-61) and Il11 (interleukin-11) are involved in the pathogenesis of VILI. Some of the results of these studies have been previously reported in the form of an abstract (16).

Methods

Word count: 499

Isolated, perfused and ventilated mouse lung

The lungs were prepared, perfused and ventilated as described (17). In brief, 9 Balb/c mice (22-30g) were ventilated with negative pressure for 1 hour with an end-expiratory pressure of -3 cm H_2O and an end-inspiratory pressure (EIP) of -10 cm H_2O. Subsequently, they were randomized to 3 groups and ventilated for the next 3 hours (n=3/group). Control: ventilation with -10 cm H_2O EIP; LPS: ventilation with -10 cm H_2O EIP in the presence of 3 mg/kg Escherichia coli bacterium lipopolysaccharide; Serotype O127: BO (Sigma, St. Louis, MO) in the perfusion buffer; OV: overventilation with -25cm H_2O EIP. At the end of the experiment, the lungs were harvested, snap frozen and stored at -80C. Total RNA and protein were extracted from these tissues.

Microarray analysis

Total RNA was extracted from lung tissue with Trizol (Invitrogen, Carlsbad, CA) (18). Labeled cRNA was generated and hybridized to Codelink Uniset I Bioarrays as recommended by the manufacturer (Amersham Biosciences, Piscataway,

NJ) and previously reported by us (13). All arrays were average scaled to the same value. For analysis we filtered out genes that did not pass manufacturer recommendation for quality control. A total of 5612 valid genes were obtained for analysis. Statistical analysis was performed by Significance Analysis of Microarrays (SAM, Stanford, CA) program and Scoregene software package (Scoregene Package, available at http://compbio.cs.huji.ac.il/scoregenes/). In SAM, 200 permutations of the data were generated and significant differences between treatment groups were determined with t-tests (p-value<0.05). The False Discovery Rate (FDR, q-value<0.05) method was applied to correct for multiple testing (19). Additionally we used the non-parametric Threshold Number of Misclassification score (TNoM) (20). Genes with TNoM=0 were considered significantly different. There was a 90% overlap between the genes identified with the two programs. A total of 567 significant genes were found.

Real-time TaqMan PCR

See details in online data supplement.

QRT-PCR was performed for 5 genes whose expression was significantly increased with overventilation and LPS treatment. TacMan PCR was executed as described previously (21). Commercially available Assay-on-Demand primer probe sets (Applied Biosystems, Foster City, CA) were used for Il11: Mm00434162_m1, Areg: Mm00437583_m1, Akap12: Mm00513511_m1, Nur77: Mm00439358_m1, and Cyr61: Mm004487498_m1. Kruskal-Wallis test was performed for multiple group comparison and inter-group differences were analyzed with Wilcoxon Rank Sum Test with SPSS statistics software (SPSS, Chicago, IL). The significance level was set at $p<0.05$.

Tissue immunohistochemical-staining

Lung tissues were fixed with 2% para-formaldehyde, merged in 30% sucrose overnight and snap frozen. Sections of lung were prepared and immunostaining was performed as described (15). Amphiregulin goat polyclonal IgG primary antibody was used (Santa Cruz Biotechnology, Santa Cruz, CA). A representative picture per group was taken with an Olympus BX51 Fluorescent microscope (Olympus America, Melville, NY).

Western blotting

Protein was extracted from whole lung tissue as described previously (11). The total protein concentration was determined with a Coomassie Plus 200 Protein Assay (Pierce, Rockford, IL). Western blot analysis was carried out as previously described for amphiregulin (22). The antibodies were the same as used for immunostaining.

Results

Gene expression profiling in a mouse model of isolated perfused and ventilated lung

We compared the gene expression patterns of -10 cm H_2O EIP ventilated (controls), -25 cm H_2O EIP ventilated (overventilation) and LPS-treated animals by microarray analysis. Figure 1 demonstrates that the lungs of the animals in the three treatment protocols exhibited distinct gene expression profiles. We identified 3 clusters of genes that behaved different in the 3 treatment groups. The expression of cluster A genes was decreased in the overventilated animals (OV) and unchanged in the LPS-treatment group (LPS). These genes show a generally lower expression.

Cluster B genes displayed decreased expression in the OV group but their expression was increased in the LPS group. Cluster C genes were increased by OV treatment. Many of them were also increased in the LPS group but to a lesser extent. Using two different statistical programs (Scoregene TNoM=0, SAM p-value<0.05) we identified 567 genes that had significantly different expression levels following LPS and/or OV treatment when compared to controls. For the complete list of genes see Table E1 in the online data supplement. A total of 182 genes were changed by OV and not by LPS. The expression of 36 genes was induced, and that of 146 genes decreased. Table 1 contains the list of well-known genes modulated only by OV. Included were genes of general metabolism, growth factor related genes, intracellular transport, one cytokine ligand and one antioxidant. The genes with decreased expression featured growth factor-related genes, cell cycle regulators, antioxidants, oncogenes, complement and coagulation related genes. Both LPS and OV regulated Three hundred eighty five genes. Among these the expression of 246 was increased and that of 139 decreased. Well-known genes that were affected by both LPS and OV are listed in table 2, among those many are cytokines, transcription factors and apoptosis-related genes. Certain growth factor genes also appear, such as granulocyte-macrophage colony stimulating factor (GM-CSF) as well as nuclear receptor subfamily 4, group A, member 1 (Nur77). LPS and OV reduced the expression of certain well-known protein kinases, cell cycle regulators, transcription factors and growth factors.

Functional grouping of genes following lung injury

We assessed cellular functions that were modified by LPS treatment and/or overventilation. First we identified genes with increased or decreased expression by creating $\log_2$ base ratios of the gene expression results (lps/c, ov/c and ov/lps

respectively). Following we used GeneXpress (http//: www.genexpress.stanford.edu) to determine whether a specific cellular function annotations were enriched in one or more of the pairwise combinations (23). Only statistically significant enriched annotations are shown in figure 2. Statistical significance was determined using hypergeometric model and corrected for multiple testing using FDR methods (13). A 95 % confidence interval was set for the FDR correction to correct for multiple testing. Findings were visualized on a color map where orange represents the up-regulation of gene expression related to the cellular function and blue stands for reduced expression in groups of genes related to the same function (Figure 2). Genes induced by OV but not by LPS were enriched for genes involved in cytoskeleton and cell communication. Genes induced only by LPS were enriched for genes involved in cytokine activity and in nucleic acid binding. Genes induced by both OV and LPS were involved in the immune response, carrier activity, and protein binding physiological processes (Figure 2). The list of cellular functions with the corresponding p-values and the number of statistically significant genes per functional annotation found among the 5612 valid genes is included in the online data supplement (Table E2).

Genes with significant changes in expression

Based on gene clustering and gene scoring results we decided to validate the RNA expression of 5 target genes: Areg, Akap12, Cyr61, Nur77 and Il11. These genes are all found in cluster C (Figure 1) with significantly increased expression (Scoregene TNoM=0, SAM p-value<0.05) following overventilation and LPS-treatment (Figure 3). Table 3 contains the gene expression fold changes in the LPS and OV treatment groups when compared to controls. The increased mRNA expression of the 5 genes was validated by RT-PCR (Figure 4). Our findings were

further substantiated by immunoblotting for amphiregulin. Amphiregulin protein expression was increased in the OV group when compared to the control and LPS treatment groups (Figure 5). Finally, we used the same antibodies to immunostain the lungs of the 3 experimental groups (Figure 6). Lungs subjected to overventilation showed increased amphiregulin staining compared to controls and LPS-treated lungs with primarily epithelial localization.

Discussion

The pathology of ventilator-associated lung injury (VALI) is not well understood. Cumulating experimental and clinical evidence suggests that this condition is triggered by mechanical overstretch of the alveoli, frequently facilitated by other causes of acute lung injury such as microbial agents (5-9). However, the extent to which overstretch and microbial agents activate similar or separate injury pathways it is not yet established. We approached this problem by comparing the gene expression profiles of overventilated and LPS-treated mouse lungs. We identified 567 genes whose expression was modified by overventilation and/or LPS. Many genes, in particular those that code for cytokines, growth factors and apoptosis-related proteins were altered both by overventilation and by LPS suggesting the presence of common lung injury pathways. However, the expression of some genes including growth factors, metabolizing enzymes, mediators and cytoskeletal proteins were modified by alveolar distention only. It is tempting to speculate that these genes activate mechanisms different from the well-known lung inflammatory pathways that have been established for microbial infections.

The largest group of genes affected by overventilation was related to apoptosis. Epithelial apoptosis has been observed in both *in vitro* and *in vivo* models of VILI (24,25). The genes identified in this study include genes involved in the FAS ligand and the caspase pathways. Among the affected genes, many are expressed in nervous system pointing to a possible neuro-inflammatory axis. For example, expression of the neuropeptide preprotachykinin-1 was related to acute lung in injury in a mouse VILI model (26). Also, Hayashi and colleagues have reported that in patients with spinal muscular atrophy, mechanical ventilation appears to contribute to neurodegeneration (27).

Validation of the data and comparison to previous studies

Microarray analysis generates a huge amount of data and hence validation is an important part of the analysis. In the present study the expression pattern of 5 genes was further substantiated by RT-PCR analysis and in addition that of amphiregulin on the protein level. Another form validation is possible by comparison to previous studies. The isolated perfused mouse lung model is well characterized and production of interleukin-6 (IL-6), tumor necrosis factor (TNF), GM-CSF (unpublished data), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), cytokine-induced neutrophil chemoattractant (KC) and macrophage inflammatory protein-2α (MIP-2α) in response to OV has been documented (7). According to the gene array the induction of these genes by OV in relation to controls was MCP-1 (Ccl2) 8.8-fold, MIP-1α (Ccl3) 6.6-fold, IL-6 5.3-fold, KC (Cxcl1) 2.9-fold, GM-CSF (csf2) 2.04-fold, and TNF 1.2-fold (MIP-2α was not present on the chip). Thus, the protein release data are well corroborated by the gene expression data, although due to the small number statistical significance was not reached in all cases. The low expression of TNF in the array is explained by the

fact that this gene is only transiently expressed after OV and is already on the decline after 180 min (28).

So far, only two gene array studies on ventilator-induced gene expression have been published (29,30). Grigoryev *et al*. analyzed lung tissue from mice ventilated *in vivo* for 2h with 15 ml/kg. Of the 69 genes for which they provide detailed data (their supplementary table 4), 62 were also detected by our analysis. The correlation for the mouse data between these two studies is highly significant (Rho = 0.57, p = 0.02). See online supplement table E3 and figure E2. While in both studies only few genes reached statistical significance if analyzed within the context of the array, taken together these two studies provide a robust set of genes induced by overventilation including amphiregulin, activation transcription factor 3 (ATF3), MCP-1 (Ccl2), tissue factor (F3), growth arrest and DNA-damage-inducible 45A (GADD45A), glutamate-cysteine ligase, heat shock protein A8 (HSP A8), IL-6, urinary plasminogen activator receptor and cyclooxygenase-2. Consistent down-regulation was observed for the adrenomedullin receptor, TCF21 (transcription factor 21) and TFF2 (recoil factor 2). Further studies on these proteins should provide deeper insight into the mechanisms of VILI.

Of interest is the observation that the correlation between the two mouse studies and the rat study was only poor. This may be explained either by the longer duration of the rat experiments (5h) or by species differences. Nonetheless, most of the robust genes listed above appear to be similarly affected by overventilation in rats. Copland and colleagues studied overventilated rats for 30 min in what is now sometimes called a macroarray (590 genes) (30). In accordance with our findings these authors observed increased expression of B-cell translocation gene 2 (Btg2) (upregulated 2.18-fold by OV), epidermal growth factor 1 (EGR1) (6.2-fold), and

Nur77 (2.04-fold), adding these genes to the list of candidate genes for ventilator-induced lung injury.

Differences between overventilation and LPS

Thus a number of important target genes were recognized by comparing arrays from different studies. Another approach is the use statistical methods to identify likely candidate genes within a given study. Herein we have used the combined statistical power of Scoregene and SAM. Applied to our data 90% of the significant genes identified by these procedures were identical, summing up to 567 genes regulated by overventilation and/or LPS.

Among the genes upregulated by both overventilation and LPS (Table 2) figured well-known pro-inflammatory (IL-6, GM-CSF, MIF) and anti-inflammatory (IL-10, IL-1ra) cytokines. Noteworthy cytokines upregulated by overventilation, which are new to the field of VILI, are IL-11 (confirmed by RT-PCR), macrophage migration inhibitory factor (MIF) and IL-22 (IL10-related T cell-derived inducible factor). Other extracellular factors upregulated by overventilation are amphiregulin (see below) and cysteine-rich protein 61 (Cyr61, confirmed by RT-PCR). Cyr61 binds to cell surface integrins and thereby induces intracellular signaling events, some of which relate to cell proliferation and angiogenesis (31). Moreover, we identified several factors involved in intracellular signaling pathways such as gravin (Akap12, A-kinase anchoring protein 12) and Nur77 (Nr4a1) to become activated by overventilation. Since these genes may point to relevant mechanotransduction pathways, we confirmed their expression by RT-PCR. AKAPs maintain multivalent signaling complexes by binding additional enzymes, including kinases and phosphatases to the cytoskeleton and may thus communicate cell distortion inside cells (32). Nur77, also known as NGFIB or TIS1, represents an orphan nuclear

receptor that is involved in cell proliferation and death and may act as an anti-apoptotic factor (33,34).

A selective response was observed for another set of genes that according to the microarray analysis was upregulated by overventilation but not by LPS (Table 1). This list includes Tnfrsf12a (Fibroblast growth factor regulated protein 2, Fn14), which modulates NF-κB activation and stimulates human bronchial epithelial cells to produce IL-8 and GM-CSF (35), the largely unexplored chemokine CXCL16, and the chemokine receptor CCR5. The majority of these factors has not yet been implicated in VILI and indicates the existence of unknown specific mechanotransduction pathways in VILI.

Also of note is the fact that we found that a number of genes were down regulated by overventilation. So far, gene regulation has been largely focused on upregulation of genes and it is only with the beginning of microarray experiments that downregulation gets some focus. Prominent among the genes downregulated by overventilation figure platelet-derived growth factor and hypoxia-inducible factor 1α (HIF1α).

The potential role of amphiregulin

Stretch can induce growth factors in epithelial cells (36,37). Tschumperlin and colleagues demonstrated that epithelial stretch increases epidermal growth factor receptor (EGFR) ligand shedding into the lateral intracellular space leading to intracellular signaling. The ligands bind to the EGFR that subsequently induces ERK mitogen-activated protein kinase (MAPK) activation (37). This mechanism could explain why several growth factor-related genes exhibited modified expression during overventilation.

One of them is amphiregulin. It is a polypeptide growth regulator and part of the epidermal growth factor family (38). Together with other epidermal growth factor receptor (EGFR) ligands amphiregulin binds to the extracellular domain of EGFR (39). EGFR binding leads to the activation of the ERK and the transcription factor nuclear factor-κB. The EGFR induces cell proliferation and the release of IL-8 and/or MIP-2 cytokines (40-42). Amphiregulin is expressed in lung tissue and has an essential role in lung branching morphogenesis (43). In human pulmonary epithelial cells amphiregulin secretion is induced upon exposure to tobacco smoke and fine particulate matter (42,44). Furthermore, increased amphiregulin secretion also contributes to GM-CSF release (44). In a mouse model of chronic asthma, ovalbumin-challenged animals displayed increased amphiregulin immunostaining of epithelial cells (45). Our immunostaining shows stretch-induced expression of amphiregulin in epithelial cells, which are exactly those cells in which ERK is activated during overventilation. The possibility that amphiregulin mediates some of the signaling responses during overventilation merits further investigation.

Taken together, our findings suggest that overventilation activates specific cellular programs that clearly distinguish it from LPS. In the future it will be important to further characterize these pathways, which may finally allow to specifically interfere with the side effects of mechanical ventilation.

Acknowledgements

We would like to thank Dr. S. Watkins and S. Alber for their technical support.

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Figure legends

Figure 1. Hierarchical clustering of 5612 valid genes. The three groups of animals exhibited distinct gene expression patterns. Each narrow column represents one animal. The animals treated in the same fashion are grouped in bigger columns and marked control (c), E. coli lipopolysaccharide treatment (lps) and overventilation (ov). N=3 animals/groups. The same abbreviations are used in all figures. Every row represents one gene. A gene is colored orange if its expression was increased. It is colored blue if the gene expression was decreased. Genes in gray did not show changes in expression. The last bar on the right shows the direction of gene expression changes (up or down) following ventilation expressed in an ov/c ratio format. Cluster A, B and C represents genes that behaved similar following one treatment but displayed different patterns when the 3 treatment groups were compared. The visualization of the analysis was done with a GeneXpress program.

Figure 2. Functional grouping of 5612 valid genes. Gene expression patterns are shown in $\log_2$ base ratios of treatment groups: lps/c, ov/c and ov/lps respectively. If the ratio is greater than 0 the small square is orange and if less than 0, it is blue. The square is colored gray if there was no significant change in the ratio of expression. Every row represents an important cellular function. Statistical significance was determined using hypergeometric model and corrected for multiple testing using FDR methods ($p<0.05$). Visualization and statistics: GeneXpress program.

Figure 3. Gene expression profiles of 5 target genes in ventilator-induced lung injury. Each square represents an individual animals treated according to control, LPS or overventilation treatment regiment. AREG= amphiregulin, AKAP12= A protein anchor protein, NUR77= nuclear factor subfamily 4, group A, member 1, CYR 61= cystein-rich protein-61, IL-11= interleukin-11. The same abbreviations will be used in the following figures. The color-coding is identical to Figure 1. Visualization: GeneXpress program.

Figure 4. mRNA expression by RT-PCR. (A) amphiregulin (AREG) (B) A protein anchor protein (AKAP12) (C) Nuclear factor subfamily 4, group A, member 1 (NUR77), (D) cystein-rich protein-61 (CYR61), (E) interleukin-11 (IL-11) expression was significantly increased following overventilation when compared to control. Values represent mean ±SD. * represents statistical significance of OV versus C, $p<0.05$.

Figure 6. Amphiregulin protein expressions. Amphiregulin protein expression increased following overventilation when compared to LPS and control treatments.

Figure 7. Immunhistochemical-staining of lung tissue. Lung sections were stained for amphiregulin. Red stain: immunofluorescent-labeled amphiregulin antibody. Blue stain: nuclear staining. 40-fold magnification. Overventilation led to increased staining when compared to LPS and control treatments.

Table 1 Genes regulated only by overventilation

Fold change units: $\log_2$ov/c

GeneBank ID	Gene name	Fold change	GeneBAnk ID	Gene name	Fold change
NM_013749	FGF regulated protein 2	3.42	NM_013685	Transcription factor 4	-2.88
AA105755	Na/K transporting ATPase	1.13	NM_008808	Platelet-derived growth factor	-1.33
NM_017472	Sorting nexin	1.16	NM_008929	Protein kinase, IFN-inducible	-2.38
*NM*_011345	Selectin (endothelial cell)	0.82	NM_008483	Laminin β2	-1.84
NM_01080	Matrix metalloproteinase 3	2.09	NM_010431	Hypoxia-inducible factor 1α	-2.49
NM_023158	CXC chemokine ligand 16	1.15	NM_007637	Chaperonin subunit 5	-2.01
NM_009941	Cytochrome C oxydase	0.77	NM_007512	ATPase inhibitor	-2.13
NM_019549	Pleckstrin	1.189	NM_009429	Tumor protein, transl. controlled	-2.26
NM_ 009917	Chemokine (C-C) receptor 5	1.26	NM_007610	Caspase 2	-2.3
NM_010637	Kruppel-like factor 4	1.36	NM_010406	Hemolytic complement	-2.64
NM_011607	Tenascin C	1.86	NM_009779	Complement component 3A	-1.33

Table 2 Genes regulated by overventilation and LPS

Fold change units: log_2ov/c

GeneBank ID	Gene name	Fold change	GeneBank ID	Gene name	Fold change
NM_019713	RAS-associated domain family 1	3.14	NM_021420	serin/threonin protein kinase 4	-2.01
NM_008655	GADD 45 B	2.88	NM_013546	Heme binding protein 1	-1.29
NM_010798	Macrophage migration inhib.factor	0.89	AF064749	Type VI collagen α subunit	-3.5
NM_009805	CASP8 and FADD-like apoptosis regulator	2.31	NM_022435	Trans acting transcription factor	-1.7
NM_009704	Amphiregulin	3.19	NM_009697	Nuclear receptor NR 2F2	-3.76
NM_031167	IL-1 receptor antagonist	1.8	NM_010406	Hemolytic complement	-2.64
NM_010516	Cystein rich protein 61	1.63	NM_033322	Small inducible cytokine family B	-2.99
NM_031185	A kap12	3.3	NM_009069	RAS-like protein RIT	-2.7
NM_010444	Nur77	1.42	NM_007993	Fibrillin 1	-1.53
NM_011337	Small inducible ctokine A3	2.73			
X02333	GM-CSF	1.4			
NM_031168	Interleukin 6	2.21			
NM_008350	Interleukin 11	2.01			
NM_016971	IL10-related T cell derived inducible factor	2.3			
NM_010548	Interleukin 10	1.19			

Table 3 Gene expression fold changes is 5 target genes in acute lung injury
The genes are selected from 567 genes with TNoM score=0, SAM p-value<0.05

Gene name	fold change $\log_2$ lps/c	fold change $\log_2$ npv/c
Amphiregulin	1.4	3.19
A protein anchor protein (gravin) 12	2.03	3.3
Nuclear receptor subfamily 4, A, member 1	0.2	1.4
Cystein rich protein 61	0.217	1.63
Interleukin 11	0.89	2.01

Figure 1

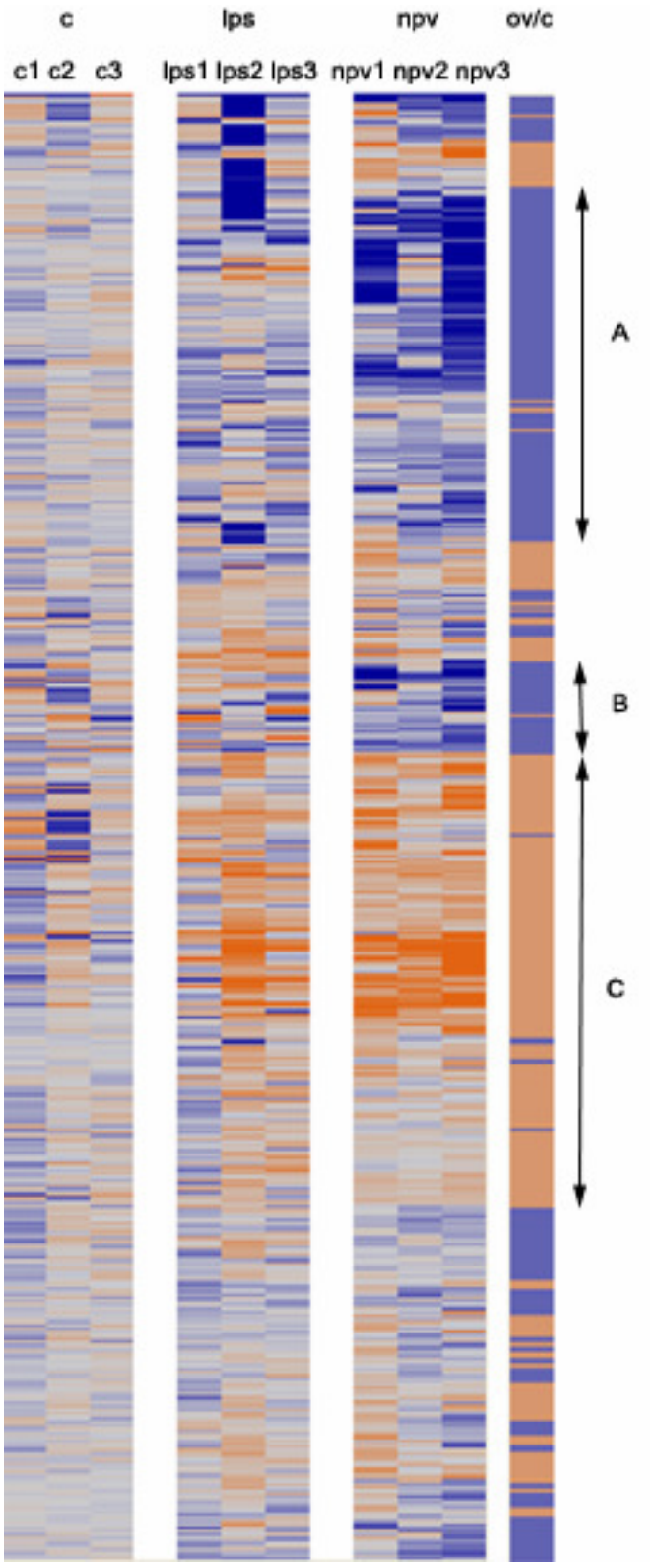
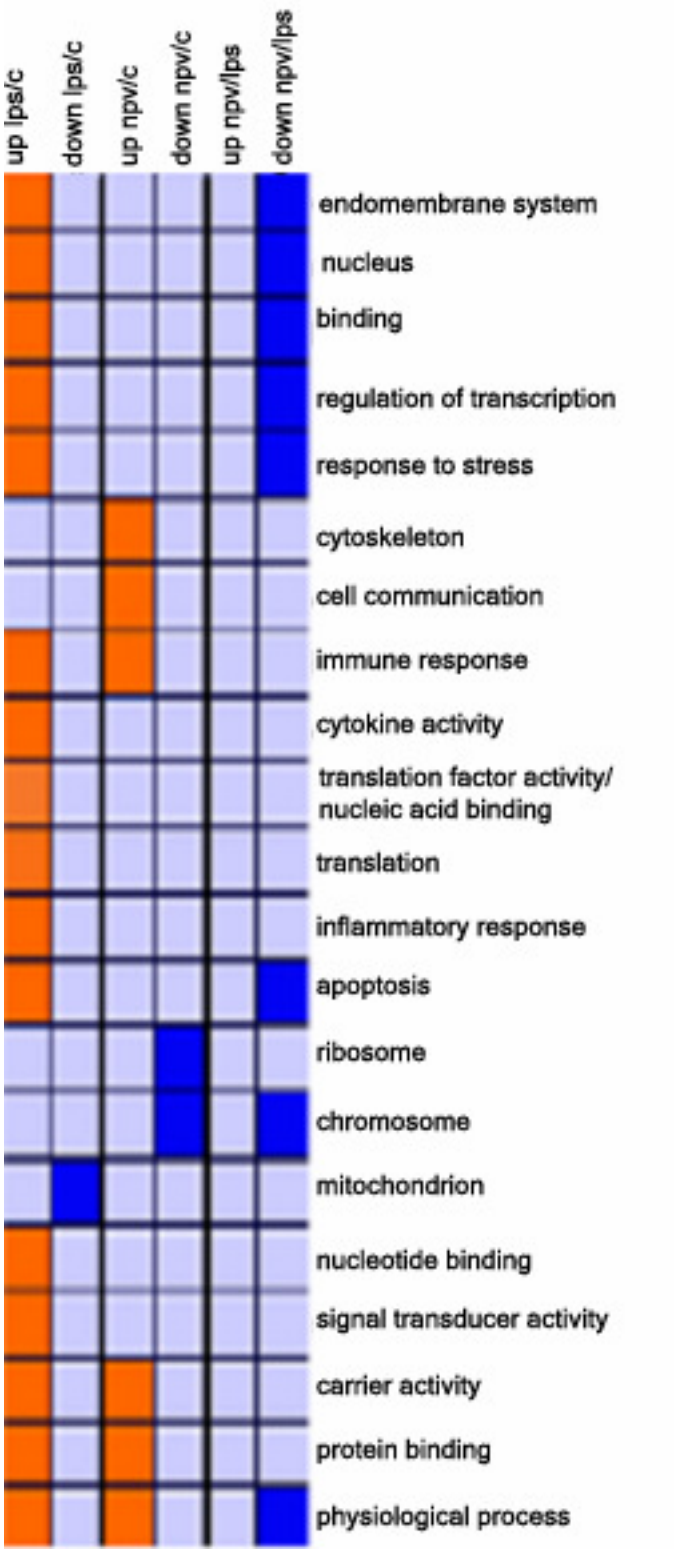


Figure 2


up lps/c
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nucleus
binding
regulation of transcription
response to stress
cytoskeleton
cell communication
immune response
cytokine activity
translation factor activity/
nucleic acid binding
translation
inflammatory response
apoptosis
ribosome
chromosome
mitochondrion
nucleotide binding
signal transducer activity
carrier activity
protein binding
physiological process

Figure 3

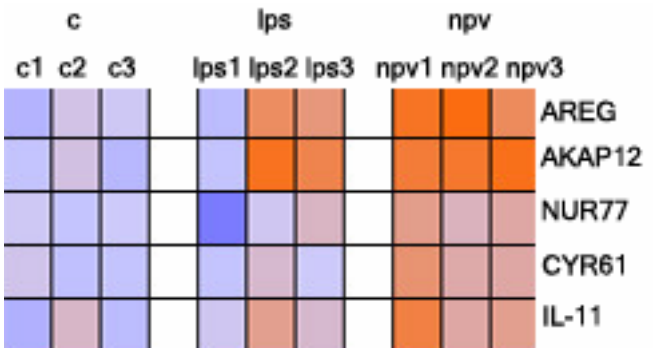
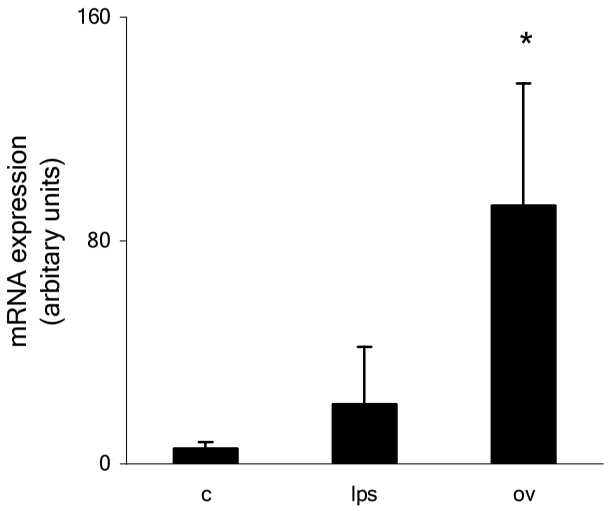
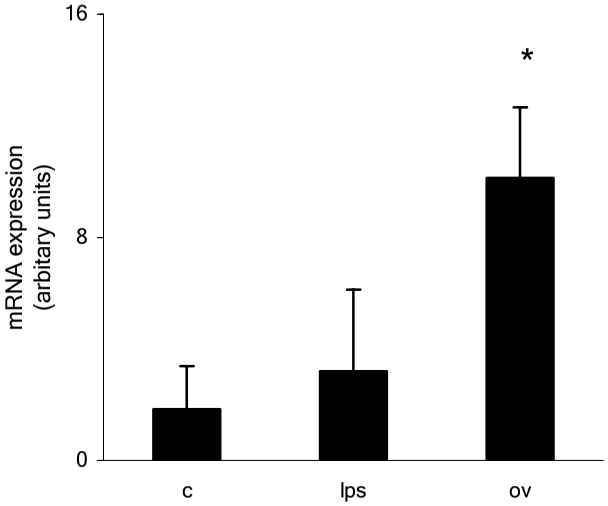

c
lps
npv
c1 c2 c3
lps1 lps2 lps3
npv1 npv2 npv3
AREG
AKAP12
NUR77
CYR61
IL-11

Figure 4

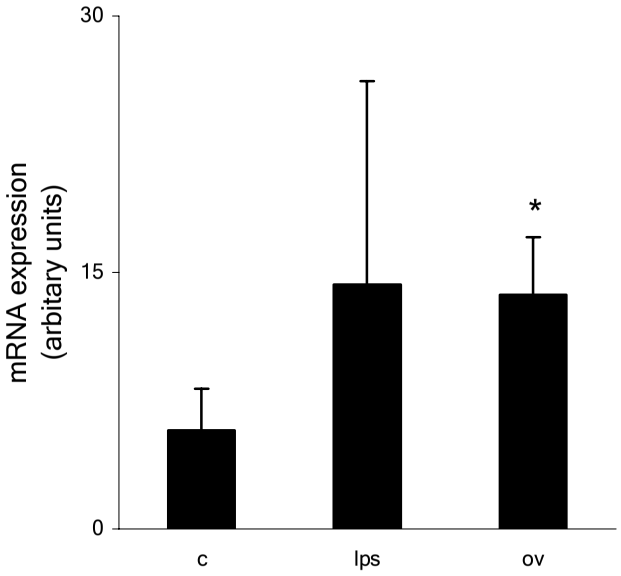
A


160
80
0
mRNA expression
(arbitary units)
*
c
lps
ov

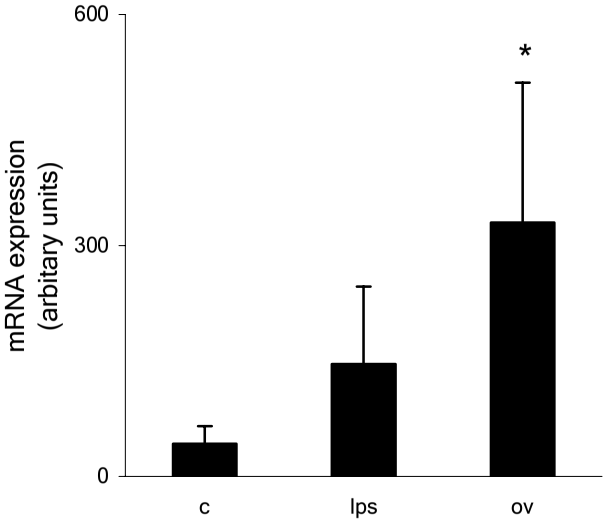
B


16
8
0
mRNA expression
(arbitary units)
*
c
lps
ov

C


30
15
0
mRNA expression
(arbitary units)
*
c
lps
ov

D


600
300
0
mRNA expression
(arbitary units)
*
c
lps
ov

E

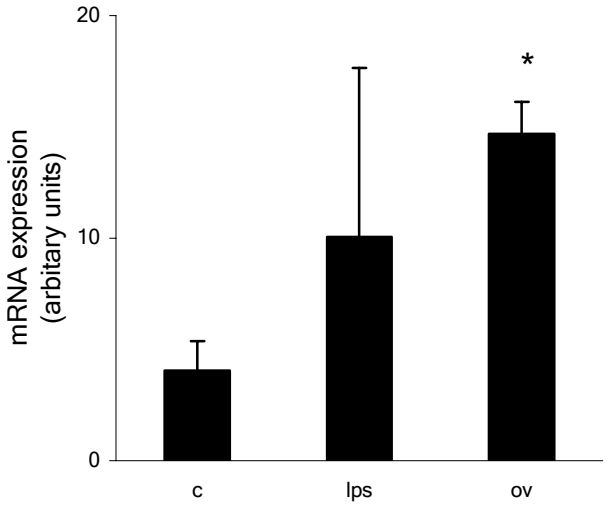


Figure 5

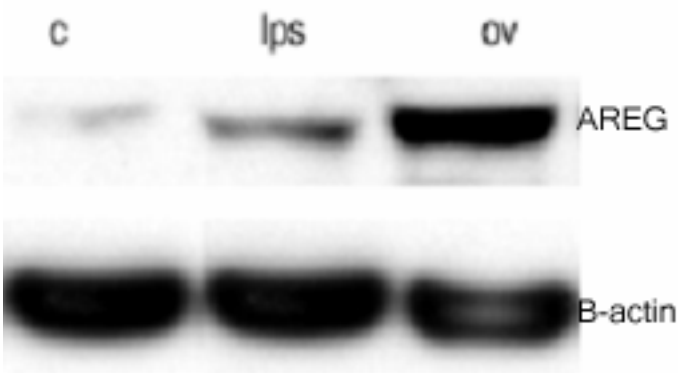


Figure 6