Acute lung injury in children: from viral infection and mechanical ventilation to inflammation and apoptosis
Bern, R.A.

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ACUTE LUNG INJURY IN CHILDREN:
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to inflammation and apoptosis

ACUTE LUNG INJURY

APOPTOSIS

Reinout A. Bem
ACUTE LUNG INJURY IN CHILDREN: from viral infection and mechanical ventilation to inflammation and apoptosis
COLOFON

Thesis, University of Amsterdam, The Netherlands
Acute lung injury in children: from viral infection and mechanical ventilation to inflammation and apoptosis

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Cover illustration by Reinout A. Bem: a commonly used metaphor for ‘apoptosis’ (derived from ancient Greek apó meaning away or from, and ptosis meaning falling down) is the seasonal event of leaves falling down from trees.
Lay-out by Chris Bor, Amsterdam.
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ACUTE LUNG INJURY IN CHILDREN: from viral infection and mechanical ventilation to inflammation and apoptosis

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PROMOTIECOMMISSIE

Promotor: Prof. dr. H.S.A. Heymans

Co-promotores: Dr. A.P. Bos
               Dr. J.B.M. van Woensel

Overige leden: Prof. dr. M.J. Schultz
               Prof. dr. A.J. van Vught
               Prof. dr. W.M.C van Aalderen
               Prof. dr. T.W. Kuijpers
               Prof. dr. P.J. Sterk

Faculteit der Geneeskunde
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Introduction and Thesis Outline
I. HISTORICAL PERSPECTIVE

This dissertation describes studies on the role of pro-apoptotic pathways in acute lung injury in children, specifically focusing on respiratory syncytial virus infection and mechanical ventilation. To introduce these terms I will start with a historical perspective.

1.1. Acute lung injury

In 1967, Dave G. Ashbaugh and co-workers wrote up a case series on their experiences with twelve patients with massive lung damage initiated by a wide variety of unrelated insults including trauma, shock and hemorrhagic pancreatitis. They describe they literally felt ‘something’ was different in these patients who required mechanical ventilation with high inflation pressures to restore oxygenation, and showed heavy edemic lungs with hyaline membrane formation and defective surfactant function at autopsy. In addition, they were surprised to see that application of positive end-expiratory pressure (PEEP) improved the oxygenation in most patients, although in the end seven of the twelve patients died. Their case series, published in the Lancet (after it was rejected three times), became a landmark article on what later was called the adult respiratory distress syndrome (1971). However, it was soon recognized that patients of all age, including children and newborns, could develop this disease, and as such, its name was changed into acute respiratory distress syndrome (ARDS), recognizing its relevance for pediatric medicine as well. In retrospect, WWII military surgeons, including Mayor Lyman A. Brewer of the Second Auxiliary Surgery Group, had already found that lungs develop increased fluid content in traumatic war casualties and they had called this the ‘wet lung’ (1946). Later, during the Vietnam War, ‘DaNang lung’ or ‘shock lung’ described similar lung pathology after trauma.

The early clinical descriptions of ARDS showed that the acute hypoxemic respiratory failure may develop following a number of different disorders including sepsis, pneumonia, aspiration and trauma. However, in the 80s and early 90s, autopsy examinations of patients with such different etiologies of ARDS revealed that these patients had highly similar lung tissue alterations, which we now consider to be the hallmarks of ARDS: diffuse alveolar damage with neutrophilic alveolitis, vascular congestion, hemorrhage, microthrombi, and intra-alveolar serum protein precipitations, such as hyaline membranes, or protein rich edema fluid. Interestingly, the characteristic eosinophilic hyaline membranes, composed of fibrin depositions and cellular debris against denuded basement membrane, had long before already been described in the respiratory distress syndrome (RDS) (or hyaline membrane disease) in preterm infants.

In 1994 the first American-European Consensus Conference on ARDS took the task to create a clear definition of ARDS, ultimately to improve knowledge and treatment of this severe lung disease. Acute respiratory distress syndrome was designated a severe form of acute lung injury (ALI), with ALI/ARDS being a syndrome of severe lung inflammation.
and increased lung permeability leading to acute hypoxemic respiratory failure. A criteria system entirely based on clinical signs for ALI/ARDS was proposed:
- acute onset
- bilateral infiltration on chest radiograph
- pulmonary-artery wedge pressure of < 19 mmHg or the absence of clinical evidence of left atrial hypertension, and
- oxygenation anomaly: PaO₂ / FiO₂ ≤ 300 mmHg (ALI) or PaO₂ / FiO₂ ≤ 200 mmHg (ARDS).

Since 1994 there have been several large prospective, randomized intervention trials by the National Heart, Lung, and Blood Institute, National Institutes of Health ARDS Network (www.ardsnet.org). However, so far these trials have resulted in very limited progress in treatment options, and at the present time ALI/ARDS remains one of the biggest challenges in adult and pediatric critical care medicine 14;15.

I.2. Respiratory syncytial virus
A specific and frequent cause of ALI/ARDS in young children is lower respiratory tract infection by respiratory syncytial virus (RSV) 16;17. RSV is a negative-sense, enveloped RNA virus of the family Paramyxoviridae, subfamily Pneumovirinae and genus Pneumovirus. It was first isolated from a group of sneezing, coughing chimpanzees at the Walter Reed Army Institute for Research in Washington DC in 1956, and was appropriately named: chimpanzee coryza agent (CCA) 18. Original CCA cultures induced similar upper respiratory symptoms in other chimpanzees, but not in guinea pigs or rodents. Interestingly, CCA appeared to be able to spread to laboratory workers who handled infected chimpanzees. A year later, Chanock et al. isolated a viral agent highly similar to CCA from two infants with lower respiratory tract infection 19;20. Because of the tendency of this ‘new’ CCA-like virus to form syncytia in HeLa cells in vitro, the virus was then renamed into respiratory syncytial virus. A study by Beem et al. with nasopharyngeal cultures from 41 young children suffering from clinical respiratory symptoms ranging from mild to severe cases determined RSV as the principal etiological agent, and formed the beginning of recognition of RSV as the leading pathogen in respiratory tract infections in children 21.

I.3. Mechanical ventilation: the beginning of (pediatric) intensive care
Breathing or ventilation is essential to human life. It involves flow of air into the lungs (inspiration) initiated by negative intrapulmonary pressure upon active expansion of the thoracic cavity, and ends with airflow out of the lungs (expiration) due to respiratory muscle relaxation and elastic recoil. The oldest references to the act of taking over ventilation by blowing air mouth-to-mouth, and thus creating positive pressure driven airflow into the lungs, date from Egyptian mythology, Bible texts and the Greek physician Galen 22. In the first printed book on pediatric diseases Libellus de egritudinibus infantium (1472) the Italian

1 human cell line from cervix carcinoma from Henrietta Lacks, † 1951.
physician Paulus Bagellardus describes neonatal resuscitation: ‘If she find it warm, not black, she should blow into its mouth, if it has no respiration, or into its anus’, although the latter suggestion appears somewhat dubious in the context of artificial ventilation. In the sixteenth century several descriptions of experimental thoracic procedures in animals refer to tracheotomy, such as by Vesalius in De Hucani Corporis Fabrica in 1543: ‘But that life may be restored to the animal, an opening must be attempted in the trunk of the trachea, into which a tube of reed or cane should be put; you will then blow into this, so that the lung may rise again and take air.’ In the 1700s and 1800s around the time of the discovery of carbon dioxide and oxygen resuscitation after drowning or mining accidents occurred by positive pressure ventilation with a bellow, or later with pistons, and first descriptions of endotracheal intubation appear.

Major progress in the application of prolonged mechanical ventilation was made at the time of poliomyelitis epidemics in Europe and the United States in the 20th century. For example, from the late 20s physicians treated children suffering from polio paralysis with Drinker’s iron lung, a tank in which a patient lay from neck to toe and in which a negative pressure environment around the chest created airflow into the lungs. In 1952 Copenhagen’s Blegdams Hospital was overwhelmed by patients with acute respiratory failure during a major polio epidemic. It was there that tracheotomy followed by intermittent manual ventilation with a rubber bag (handled by medical students) was performed on a wide scale. The organization of wards specialized in prolonged artificial ventilation during which patients could recover from this disease formed the beginning of intensive care units (ICUs). Although initially adult ICUs focused primarily on the respiratory system, pediatric ICUs (first established in 1955) followed the model of neonatal ICUs in which specialized generalists managed patients of a specific age, suffering from a wide variety of critically ill diseases, including different organ systems. The clustering of critically ill patients in such specialized wards was later also recognized to be highly suitable for post-surgery patients, extending the field of intensive care medicine. Today, machines have taken over the ventilation of ICU patients and modern positive pressure mechanical ventilator devices come in many types, designed to control volume, pressure and frequency adapted to the patient’s breathing activity.

1.4. Apoptosis
In the organogenesis and development of living multicellular organisms the elimination of cells by cell death is as important as cell proliferation. One of the most popular examples highlighting the importance of cell death in the development of embryonic tissues is the ‘sculpting’ of our hands and fingers by the elimination of cells located in between digits. Detailed work derived from the 2002 Nobel Prize winners Sydney Brenner, H. Robert Horvitz and John E. Sulston showed that of the 1090 somatic cells of the nematode Caenorhabditis elegans hermaphrodite 131 are fated to die during development into adulthood, underlining the extent of cell death in this early stage of life. In addition, cell death counterbalances...
cell proliferation and thereby prevents excessive growth of tissues or tumor development, which is essential for normal homeostasis.

Since the initial concept of cell death, as is discussed in Rudolph Virchow’s lectures on cellular pathology in 1858, numerous morphologic descriptions using a broad terminology have been proposed in an attempt to define the complex process of a dying cell. ‘Apoptosis’, derived from Greek (apó meaning away or from, and ptosis meaning falling down), refers to a distinct type of cell death morphologically characterized by cell shrinkage (as opposed to swelling, oncosis), and enhanced cell density. Other prominent features of an apoptotic cell include the pyknotic condensation of the chromatin, fragmentation of the nucleus (karyorhexis) and budding, which describes the emission of cellular processes containing intact organelles and nuclear fragments. The term ‘apoptosis’ was introduced by J.F. Kerr in 1972 after having studied cell death in toxic liver injury by electron microscopy for almost 10 years. However, throughout history, terms such as ‘karyolysis’ (1879), ‘chromatolysis’ (1885), ‘shrinkage necrosis’ (1965) and ‘programmed cell death’ (1965) have preceded the term ‘apoptosis’ and described more or less similar morphologic observations.

After the early morphological definition of apoptosis there has been a surge of research focusing on the actual biochemical and genetic mechanisms implemented in this type of cell death. At the beginning of this, in 1986, the first genes recognized to be involved in the apoptotic process were bcl-2 and ced-3 and -4. Bcl-2, cloned from a follicular lymphoma cell line, was found to inhibit apoptosis upon overexpression and therefore for the first time linked the development of cancer to inhibition of cell death (in contrast to stimulation of cell proliferation). After bcl-2, many genes, such as p53, ced and bim as well as other important components such as Fas, cytochrome c and the family of caspases were implicated in the control of apoptosis. In fact, the aspect of control, embodied by the underlying genetic program and protein machinery, is now considered to be a main characteristic of apoptosis. It provides the means for rapid cell death triggered by both external and internal stimuli at any point during life and this may differ from ‘programmed cell death’, which technically refers to cell death occurring at a specific (programmed) time point. Furthermore, the suicidal program of apoptosis contrasts with ischemic or accidental cell death for which the term necrosis is widely used, although in another paradigm necrosis refers to the final morphologic appearance of a dead cell, rather than to a mechanism or process of cell death.

Currently, the field of apoptotic research is broad and one of the most popular in modern biology. Besides its pivotal role in organogenesis and development, apoptosis is now implicated in a wide variety of diseases, including cancer, autoimmune diseases, neurodegenerative disorders and numerous infectious diseases. Data accumulating over the past years has shown that unbalanced apoptosis, in regard to the extent and/or timing, forms the basis of many pathogenic processes. Intervention in the genetic
mechanism and regulatory proteins of apoptosis may therefore provide new treatments for disease.

2. ALI/ARDS

2.1. Incidence and mortality
Approximately 6% of adult patients admitted to the ICU in Western countries develops ARDS, and this is associated with a mortality rate as high as 40-55%. To grasp the impact of such numbers: it is estimated that in the United States annually more than 190,000 patients develop ALI/ARDS, of whom approximately 75,000 die. In comparison, studies have reported less than 3% of all children admitted to the pediatric ICU (PICU) develops ARDS, being fatal in up to 35% of the cases. Interestingly, studies among both adult and pediatric ALI/ARDS patients seem to confirm this positive correlation between an adverse outcome and age.

2.2. Clinical disorders
ALI/ARDS can develop following a wide variety of clinical disorders with direct or indirect lung injury, including sepsis, pneumonia, aspiration, trauma, transfusion, drowning and burns. Bacterial pneumonia and sepsis are among the most common etiologies of ALI/ARDS in both adults and children, and form an important risk factor for death. However, specific viral pathogens, such as RSV, induce ALI/ARDS in young children in recurrent seasonal outbreaks, but are associated with low mortality rates upon supportive treatment. Other risk factors for death in pediatric patients include multiple organ failure, dysfunction of the central nervous system and pre-existing immune disorders.

2.3. Treatment
Up to date, mechanical ventilation and supplemental oxygen remain the cornerstone treatments for ALI/ARDS in adults and children. Experimental treatments include the use of corticosteroids, prone positioning, high PEEP, high frequency ventilation, exogenous surfactant, nitric oxide inhalation, recruitment manoeuvres, and restrictive fluid regimes. Although several of these studies identified patient subgroups that may benefit from such strategies, so far, these have failed to show consistent improvement in primary end points such as mortality in randomized controlled trials. An exception to this was the 1996-1999 NIH ARDS Network 'ARMA' study which showed that mechanical ventilation with low tidal volume (6 ml/kg) as compared with high tidal volume (12 ml/kg) increased the survival of ALI/ARDS patients: 31% vs. 40% mortality respectively. This study provided a 'simple', beneficial and clinically relevant treatment strategy for ALI/ARDS patients which could be generally applicable by physicians.
at ICUs worldwide. However, at the same time it also highlighted the potential detrimental (iatrogenic) effects of ICU treatment on the course of ALI/ARDS, as it further proved the clinical relevance of the ventilator induced lung injury (VILI) concept, which refers to lung damage by direct physical forces as well as biological (pro-inflammatory) mediators caused by mechanical ventilation 52-54.

Remarkably, despite the ‘success story’ of the ARMA trial, Phua et al. recently reported a systematic review showing no decrease in mortality in ALI/ARDS patients over the last 10 years 15. One of the potential problems in finding effective treatment may be the relative high heterogeneity of ALI/ARDS patients with respect to etiology and underlying disorders. Furthermore, because the diagnosis of ALI/ARDS is entirely based on a set of non-specific clinical parameters, there may be a poor correlation with histopathological findings which is a potential confounding factor in treatment trials 55.

2.4. Pathogenesis

Given the lack of specific treatment and high mortality in ALI/ARDS, more insight into pathogenesis is urgently needed. Here, I will present two main pathogenic theories, the ‘neutrophil hypothesis’ and the ‘epithelial cell hypothesis’. Although the central events in these hypotheses may show considerable overlap and interaction in vivo, they help to form a conceptual framework for this thesis. It is important to note that there may be several other mechanisms that are important in ALI/ARDS pathophysiology 6, but their discussion is beyond the scope of this thesis.

In the ‘neutrophil hypothesis’ it is proposed that lung tissue damage occurs secondary to the extensive influx and activation of polymorphonuclear (PMN) neutrophils releasing a number of potentially harmful reactive oxygen- and nitrogen species and proteolytic enzymes, such as elastase, cathepsin G, and metalloproteinases 56-58. In the acute phase of ALI/ARDS neutrophils adhere to the lung capillary endothelium and migrate through the interstitium into the alveolar spaces. Studies in humans with ALI/ARDS have shown high PMN counts in bronchoalveolar lavage fluid (BALF), and marked neutrophilic accumulation in lung tissue potentially due to delayed apoptosis 8;9;59. The recruitment and activation of neutrophils is in part mediated by a number of (chemotactic) cytokines such as interleukin (IL)-1, IL-6, IL-8 and TNFα, which are released locally by macrophages and epithelial cells 6;60. Although a well orchestrated neutrophil response in the lungs is critical in the host defense against microorganisms, it is thought that dysregulation leading to enhanced neutrophil influx and activation and delayed apoptosis contributes to the tissue injury in ALI/ARDS 61. Animal models for endotoxemia- and aspiration-induced ALI/ARDS, have shown that PMN depletion or inhibition of chemotactic signaling diminishes lung inflammation and permeability 62-67. Yet in humans with normal lungs recruitment of PMNs by leukotriene B4 does not alter the lung permeability, and ALI/ARDS can develop in adults and children with profound neutropenia 68-70. Together these data suggest that
the neutrophilic response plays an important role in the development of ALI/ARDS, but that at the same time other pathogenic mechanisms can be involved as well.

The ‘epithelial cell hypothesis’ proposes that enhanced lung epithelial cell death is a key initial event in the development of ALI/ARDS. Throughout the lungs and airways a single layer of epithelial cells lines the surface between the human body and the outside environment. The epithelium acts as a physical barrier to the entry of foreign antigens or pathogenic microbes and viruses, and functions as a major source of inflammatory mediators essential for host defense. In addition, in the airways pseudostratified epithelium produces mucociliary transport which clears debris from the lumen, while in the alveoli the epithelium ensures gas-exchange (type I cell) together with surfactant production and ion transport (type II cell). Under normal conditions the permeability of the tight alveolar epithelium is low, in particular in comparison to the capillary endothelial barrier, thereby preventing plasma proteins and liquid from entering the alveolar- and airspaces. Epithelial damage is a hallmark of ALI/ARDS, and given the above mentioned diverse functions of the lung epithelium it is not surprising that injury to this cell layer can sink the patient into a spiral of negative events: epithelial injury increases lung permeability resulting in the formation of protein-rich edema in the alveolar spaces leading to impaired gas-exchange; disrupted ion-fluid transport in injured epithelial cells may impair the re-absorption of the edema; both decreased production and edema-induced inactivation of surfactant proteins and lipids further alters lung physical properties such as enhanced surface tension leading to enhanced respiratory effort; at the same time, lung pathogens or locally produced inflammatory mediators may spill over to the circulation resulting in sepsis/systemic pro-inflammatory responses (decompartmentalization) and multiple organ failure.

3. THESIS

A key focus point of the above mentioned ‘epithelial cell hypothesis’ is the activation of specific pro-apoptotic pathways in or directed against the lung epithelial cell. Early descriptive studies in humans with ALI/ARDS have shown characteristic apoptotic changes in alveolar epithelial cells, and this has led to the search of potential pro-apoptotic (soluble) mediators in the lungs, ultimately to find new therapeutic targets. The overall goal of the present dissertation is to investigate the role of several classical pro-apoptotic pathways in the development of lung epithelial injury, with special focus on pediatric ALI/ARDS, including severe RSV disease.

3.1. Outline

Chapter 1 reviews the current literature on the role of lung epithelial cell apoptosis in (pediatric) ALI/ARDS. We give an overview of several classical pro-apoptotic pathways...
implicated in the ‘epithelial cell hypothesis’. Furthermore, we discuss our hypothesis that the ongoing lung development and maturation in young children and infants may affect the susceptibility to apoptosis in ALI/ARDS and vice versa. In **chapter 2** we investigate a classical apoptosis marker in lung tissues of pediatric patients who died with ARDS.

In chapter 3-6 we investigate the role of three classical extrinsic pro-apoptotic pathways:
- **Chapter 3** describes the interplay between the ‘neutrophil hypothesis’ and the ‘epithelial cell hypothesis’ mediated by the Fas (CD95) death receptor/FasLigand (FasL) system in mice.
- **Chapter 4** describes the role of the tumor necrosis factor related apoptosis-inducing ligand (TRAIL) death receptor pathway in the ‘epithelial cell hypothesis’ in children with severe RSV disease.
- **Chapter 5 and 6** describe the role of the granzyme pathway in the ‘neutrophil hypothesis’ and the ‘epithelial cell hypothesis’ in severe RSV disease in children and a mouse model.

Finally, as discussed above, ICU treatment may be an important co-factor in the development of ALI/ARDS. Therefore, in **chapter 7** we investigate the role of mechanical ventilation in the ‘neutrophil hypothesis’ and the ‘epithelial cell hypothesis’ in severe RSV disease in a mouse model.
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Lung Epithelial Cell Apoptosis in Acute Lung Injury in Infants and Young Children

Reinout A. Bem
Albert P. Bos
Gustavo Matute-Bello
Minke van Tuyl
Job B.M. van Woensel

1 Pediatric Intensive Care Unit, Emma Children’s Hospital AMC, Amsterdam, The Netherlands; 2 Center for Lung Biology, Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of Washington, Seattle (WA)

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ABSTRACT

Apoptosis of lung epithelial cells is implicated in the pathogenesis of acute lung injury. Most research on this subject has focused on adults. Up to date, very little is known about a potential interaction of this process with lung development in children. In the present study we aimed to summarize the current literature on lung epithelial cell apoptosis and common causes of acute lung injury in infants and young children and to identify new areas of research. Overall, few studies have specifically focused on lung epithelial cell apoptosis in acute lung injury in children. Nevertheless, the limited literature suggests that this may be an important pathogenic mechanism in respiratory distress syndrome of infants (IRDS) and viral respiratory tract infection. Apoptosis is an essential process during lung development and maturation. Insufficient attention has been paid to potential consequences of this for the short and long term outcome of acute lung injury in infants and young children.
INTRODUCTION

Characteristic and common causes of acute lung injury in infants and young children include respiratory distress syndrome of preterm infants (IRDS), viral lower respiratory tract infection and ventilator-induced lung injury. Although it is generally believed that lung injury during early life may have negative consequences for long term lung function, the exact effects of acute lung injury on the developing lung are incompletely understood. Likewise, to what extent the stage of lung maturation contributes to the susceptibility to acute lung injury remains controversial. Both acute lung injury incidence and mortality rates appear to be lower in infants and young children as compared to adolescents and adults, and therefore further insight into the mechanisms that may be involved herein are of great interest.

Apoptosis, a form of programmed cell death, is a highly regulated series of events leading to the elimination of cells. It is an essential process for normal organogenesis in the child, including the development and maturation of the lungs. Lung epithelial and mesenchymal cell apoptosis has been observed throughout different stages of lung development before and after birth, and is believed to greatly contribute to gas-exchange surface formation and airway branching. At the same time, apoptosis of lung epithelium has been associated with the pathogenesis of acute lung injury. When exposed to injurious events, airway and alveolar epithelial cells can react with a broad arsenal of protective measures such as the production of inflammatory mediators and anti-oxidants, depending on the cause of injury. Alternatively, the lung cell may lose its ability to survive and may die either by necrosis or apoptosis. In this light, the process of apoptosis has originally been viewed as controlling injury and inflammation. Although indeed accumulating evidence suggests that apoptosis of the lung epithelium is an important cellular mechanism during acute lung injury, it remains a matter of debate under which conditions lung epithelial cell apoptosis is beneficial (e.g. controlling infection) or ‘out of balance’ and detrimental (e.g. acute respiratory distress syndrome, ARDS) to the patient. For example, a patient suffering from viral pneumonia could benefit from enhanced viral clearance by apoptosis of infected lung cells, but may experience severe oxygenation anomalies when widespread lung epithelium apoptosis occurs. An overshoot of lung epithelial cell apoptosis contributes to the loss of integrity of the alveolar capillary barrier function, resulting in pulmonary edema and surfactant abnormalities. During the past few years, lung epithelial cell apoptosis has been extensively studied in the context of acute lung injury. Because apoptosis is a regulated process that potentially can be intervened, unraveling the precise role and mechanisms may help find anchor points for new treatment strategies.

Up to date, research on lung epithelial cell apoptosis and acute lung injury has mainly focused on adult patients. Because one could easily argue that resting levels of apoptosis as part of the developmental stage in the lungs of young children and infants may influence both the susceptibility and consequences of acute lung injury, the role of lung epithelial cell apoptosis during acute lung injury in children deserves further exploration. In the present
study we aim to summarize the current literature on this subject to identify new areas of research in this specific age group.

MECHANISMS OF APOPTOSIS

Before going into more detail on lung epithelial cell apoptosis and acute lung injury in pediatric patients, we will present a brief conceptual framework of the cellular mechanisms of apoptosis especially focusing on acute lung injury.

Cell death in multicellular organisms occurs either by necrosis or apoptosis. Each of these two processes have distinct morphological and biochemical characteristics. Apoptosis is an active process and follows signaling through specific death pathways, as described below. In contrast, necrosis is a process that results from the inability of a cell to sustain its homeostatic mechanisms. However, a strict distinction between the two types of cell death is difficult because certain forms of apoptosis also show necrotic features, and conversely, it has been found that cells exposed to pro-apoptotic stimuli can also die due to necrosis. Apoptosis is associated with membrane blebbing, disruption of the cell into apoptotic bodies, and fragmentation of the DNA. In 'classical' apoptosis activation of a family of intracellular substrate specific proteases called caspases precedes this fragmentation. However, several caspase-independent signals of (non-'classical') apoptosis have also been discovered.

At least three major pathways may be of importance for lung epithelial cell apoptosis during acute lung injury (Figure 1). A receptor-mediated (extrinsic) pathway can be triggered by ligation of transmembrane death receptors belonging to the family of tumor necrosis factor receptors (TNFR), such as Fas (CD95) and TNF-related apoptosis-inducing ligand (TRAIL) receptor. These receptors are activated by Fas ligand (FasL) or TRAIL, either in membrane-bound or soluble form, or by TNFα. The extrinsic pathways are associated with intracellular caspase-8 activation leading to further downstream activation of other caspases, such as the main effector caspase-3, and execution of apoptosis. Elevated levels of both soluble Fasl and TNFα has been found in bronchoalveolar lavage fluid (BALF) of humans with ARDS. In addition, blocking the Fas/FasL pathway has been found to attenuate lipopolysaccharide (LPS)- and shock/sepsis-induced acute lung injury in animal models. Studies have shown that both the TNFα and FasL death receptor pathways may modulate hyperoxia/oxidative stress-induced apoptosis during acute lung injury. However, it has also been demonstrated that mice deficient for TNFR or Fas are not protected against oxygen toxicity, suggesting that these death receptor pathways are not essential in hyperoxia-induced acute lung injury.

A second pathway of apoptosis can be triggered by injurious events such as DNA damage, oxidants, radiation and intracellular calcium overload. This (intrinsic) pathway involves the release of pro-apoptotic factors, like cytochrome c from the mitochondria...
into the cytosol, and is regulated by the Bcl-2 family of proteins. Thereafter, apoptosis is triggered through both caspase-dependent and caspase-independent death pathways. There is evidence of cross-talk between the receptor and mitochondrial mediated pathway, suggesting that these two signaling routes to interact during apoptosis. Activation of the mitochondrial pathway appears to play an important role in hyperoxia-induced acute lung injury, and some studies suggest that reactive oxygen species directly induce cytochrome c release from the mitochondria into the cytosol. Finally, cytotoxic T-cells and natural killer cells exploit a pro-apoptotic pathway that is thought to play an important role in the clearance of virus-infected cells. In the classical paradigm these effector lymphocytes elicit the formation of pores in the membrane of an infected target cell by means of a protein called perforin. Subsequent

**Figure 1.** Pathways of caspase-(in)dependent apoptosis in lung epithelial cells mediated by mitochondrial disruption or death receptor stimulation initiated by recruited effector lymphocytes, neutrophils (PMN) or macrophages. Members of the Bcl-2 family including Bcl-2, Bax and Bcl-XL and p53 regulate cytochrome c release from the mitochondria in response to stimuli such as DNA damage, reactive oxygen species (ROS) or calcium overload. Cytochrome c in the cytosol assembles with apoptotic peptidase activating factor 1 (Apaf-1) to activate initiator caspase-9. Adaptor proteins interact through their death domain upon activation of the death receptor pathway by FasL or TRAIL, leading to activation of initiator caspase-8. The mitochondrial and death receptor pathway can interact through Bid. The activation of caspase-8 or caspase-9 leads to the downstream activation of the caspase cascade including effector caspase-3, -6 and -7, resulting in apoptosis. Granzymes delivered to the cytosol by effector lymphocytes can activate caspases and Bid to induce apoptosis. The inhibitor-of-apoptosis proteins (IAPs) can block several caspases thereby inhibiting cell death.
delivery through these pores of cytolytic granular proteases, known as granzymes, into the cytosol of the target cell leads to activation of both caspase dependent and independent apoptotic pathways. Upregulation of mRNAs of granzymes and perforin has been found in BALF of humans with ARDS 29.

APOPTOSIS AND PEDIATRIC ACUTE LUNG INJURY

We used a Medline database and references from identified articles to perform a literature search relating to epithelial cell apoptosis, acute lung injury and intensive care treatment in children. In this section we specifically focus on common causes of acute lung injury in infants and young children because of the importance of lung development in this age group.

Perinatal acute lung injury
The most common type of acute lung injury in newborns is the respiratory distress syndrome of preterm infants (IRDS). IRDS is primarily associated with surfactant deficiency, but other pathogenic mechanism such as an exaggerated inflammatory and pro-apoptotic response may play a role as well 30;31. Lukkarinen et al. found increased leukocyte infiltration and increased numbers of apoptotic epithelial cells in autopsy lung specimens of ventilated neonates who died from IRDS, as compared to ventilated control patients without obvious lung disease 32. Similarly, May et al. reported an increase in the apoptosis markers terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) and caspase-3 activity in alveolar epithelial cells of 27 ventilated infants with fatal IRDS, as compared to still borns 33. In addition, autopsy samples of ventilated infants with bronchopulmonary dysplasia following IRDS showed increased apoptosis of both alveolar and bronchial epithelial cells 34. However, these studies should be interpreted with caution because of their retrospective design and the very heterogeneous character of the cohorts studied. In particular, the contribution of mechanical ventilation or hyperoxia per se to epithelial cell apoptosis was not addressed in these studies. In the study of May et al. surfactant treatment seemed to reduce alveolar epithelial cell apoptosis 33, an observation in line with results described by White et al. showing that surfactant protein A (SP-A) inhibits alveolar epithelial cell apoptosis through interaction with tyrosine phosphorylation pathways downstream of the SP-A receptor 35.

Another line of research relevant to apoptosis during acute pulmonary disease in newborns has focused on meconium-induced acute lung injury. This disorder involves an acute inflammatory reaction following perinatal meconium aspiration. Several recent animal studies have shown that increased apoptosis of particularly airway epithelial cells is an important feature of meconium instilled lungs 36;37. In addition, A549 cells (a human carcinomic, alveolar basal epithelial cell line) show caspase-3 dependent apoptosis following exposure to meconium 37.
Respiratory tract infection

Respiratory tract infections, in particular of viral origin, are a frequent cause of acute respiratory insufficiency in young children. Many in vitro studies have investigated lung epithelial cell apoptosis during infection with common pediatric respiratory viruses such as respiratory syncytial virus (RSV), influenza-, rhino- and adenovirus, some of which have been reviewed extensively elsewhere. During infections with these viruses, apoptosis is considered to be a major host defense mechanism, by limiting viral spread and replication. On the other hand, viruses often display strategies to evade apoptosis. Several in vitro studies using different lung epithelial cell lines have shown apoptosis and up-regulation of pro-apoptotic gene expression such as Fas in response to respiratory viral invasion. However, it remains unclear under which conditions excessive and uncontrolled lung epithelial cell apoptosis following viral infection may contribute to acute lung injury and organ dysfunction. It is likely that intrinsic host factors, as has been suggested for asthmatic adults and children with cystic fibrosis, as well as pathogen factors determine this balance between ‘good’ or ‘bad’ apoptosis.

Worldwide, RSV is the most common cause of lower respiratory tract infection in young children. Every winter a high number of children with severe RSV infection require mechanical ventilation and many of them fulfill the criteria of ARDS. Several animal studies have highlighted the importance of apoptosis during RSV infection in vivo. Viuff et al. demonstrated that apoptosis is an important way of clearance of infected cells in a model of bovine RSV infection in calves. A recent murine study suggested a detrimental role for the Fas/FasL system during severe RSV infection. In that study, RSV-induced clinical illness was strongly reduced in mice carrying a gene encoding for a nonfunctional form of FasL, while viral clearance was moderately delayed. However, no direct evidence for lung epithelial cell apoptosis in relation to disease severity was presented. Such cytopathological events may be difficult to demonstrate in these animal models because RSV is not a natural pathogen for mice and replicates to a limited extent in murine respiratory epithelial cells.

Bacterial infection, either as the primary cause or secondary to viral infection, often leads to acute lung injury in children, comparable with adults. In adult animal models LPS-induced acute lung injury appears to be associated with widespread apoptosis of the lung epithelium. However, to our knowledge, so far no studies have focused on the role of lung epithelial cell apoptosis secondary to bacteria or bacterial products in newborn mice or children.

Intensive care treatment

Oxygen treatment and mechanical ventilation are the cornerstones of the management of critically ill patients with acute lung injury. However, it is widely accepted that both treatment modalities can cause additional injury to the lung.
Potential mechanisms of hyperoxia-induced lung injury involve the production of reactive oxygen species, release of pro-inflammatory cytokines and cell death. Several experimental studies have shown that besides necrosis, activation of pro-apoptotic pathways is a prominent feature in the cellular response to oxidative stress, although the exact contribution of lung epithelial cell apoptosis in hyperoxia-mediated lung injury in the clinical setting is unclear. Several studies have evaluated the influence of age on hyperoxia-induced lung injury and apoptosis. Auten et al. found DNA damage and oxidation, independent from Bcl-2 and Bax transcription or with caspase-6 activity, in lung parenchymal cells of newborn rats that were exposed to hyperoxia for 8 days. However, Mantell et al. found evidence for a delayed apoptotic response in lung parenchymal cells in newborn rabbits exposed to hyperoxia, as compared to their adult counterparts. Furthermore, in a recent study Mao et al. found that activation of Fas results in a protective proliferative response rather than a pro-apoptotic response in hyperoxia-treated newborn mice. Interestingly, newborns of rodents and rabbits exposed to hyperoxia have a delayed inflammatory reaction in their lungs and survive longer than adult animals. For example, treatment with 100% O₂ is lethal in adult rabbits at 72 hr exposure, while newborns live for several days thereafter with lung inflammation and edema not being evident until about 96 hr of exposure. Taken together, these findings suggest age-related differential pro-apoptotic responses may be involved in the lower susceptibility of newborns to hyperoxia-induced lung injury.

Mechanical stress leading to cellular stretch during mechanical ventilation may trigger responses that include pro-inflammatory mediator release and apoptosis by mechanotransduction in lung epithelial cells. The mechanisms responsible for ventilator-induced lung injury are being extensively investigated in order to find optimal ventilator strategies to minimize iatrogenic damage. As mentioned above, studies in mechanically ventilated preterm infants have reported increased numbers of apoptotic lung epithelial cells in vivo. However, in these studies the relative contribution of mechanical ventilation, hyperoxia and primary lung disease to the demonstrated apoptosis remains unclear. Recently, Smith et al. presented preliminary data on the role of age in lung injury induced by a combination of mechanical ventilation and LPS challenge: they found an upregulation of anti-apoptotic response genes in the juvenile mice, as compared to adult mice, suggesting a protective effect of age in this model.

**APOPTOSIS AND LUNG DEVELOPMENT**

To support our hypothesis that both outcome and susceptibility to acute lung injury might be affected by age, we will give a brief overview on the role of apoptosis in lung development.

The development of organs requires an orchestrated and complex interplay between proliferation, differentiation and apoptosis. During organ morphogenesis, apoptosis
occurs either by direct stimulation or by lack of growth and/or differentiation factors, resulting in removal of unwanted or superfluous structures. Although most organogenesis occurs in utero, several organ systems do not complete their development and maturation until after birth. In humans, alveolarization and microvascular maturation of the lungs continue up to at least a few years after birth.

Apoptosis has only recently been implicated in the process of lung development, probably related to cell stretching and under the influence of sex hormones. During gestation, mesenchymal and alveolar epithelial cell apoptosis coincides with airway branching and alveolar epithelium thinning, respectively. After birth, superfluous surfactant producing type 2 alveolar epithelial cells are removed by apoptosis and by differentiation into type 1 alveolar epithelial cells, which form most of the alveolar gas-exchange surface. In a series of elegant experiments, De Paepe et al. provide evidence that regulation of the Fas/FasL system is critical for type 2 alveolar epithelial cell apoptosis in developing lungs of both rabbits and mice.

One of the major underlying mechanisms believed to be involved in regulation of apoptosis in the lungs is cellular stretch. Just as normal breathing causes lung cell stretch after birth, fetal breathing movements and growing lung buds may generate physical strain in the developing lung. Several possible events in lung cell mechanotransduction have been suggested, such as stretch activation of ion channels, conformational change of cytoskeleton proteins (e.g. cadherins and integrins) or direct stretch dependent transcriptional regulation (e.g. upregulation of FasL). However, the precise mechanism by which lung cell stretch is linked to activation of apoptotic pathways and the potential impact of additional stress caused by mechanical ventilation is far from clear.

Based on the above mentioned observations it is very likely that resting levels of apoptosis in the lungs of children differ from those in adults, depending on the developmental stage of the lungs. High or low resting levels of both extrinsic and intrinsic pro- and anti-apoptotic factors may affect the susceptibility of the lung to injurious events. Whether such an influence is advantageous or detrimental to the outcome cannot be known unless more research specifically focuses on this subject. Up to date, this has been given insufficient attention to in the literature. In addition, more insight is needed into the potential long-term effects of dysregulation of apoptosis during acute lung injury on lung function. Although research evaluating the long term consequences of acute lung injury in children is very scarce, some reports show both restrictive and obstructive disease in a substantial part of children who have suffered from ARDS. Long term effects of apoptotic imbalance during organ development mediated by injury have been suggested for the brain and kidneys. Interestingly, De Paepe et al. showed that FasL expression induced by a transgenic tetracycline expression system in airway and alveolar epithelial cells in mice during perinatal lung development results in disrupted alveolarization associated with increased apoptosis. In addition, Dieperink et al. showed that hyperoxia treatment of fetal mouse lung explants results in decreased
airway branching, possibly mediated by increased apoptosis. These findings support the hypothesis that disruption of the delicate interplay between apoptosis and proliferation during acute lung injury in early childhood may influence normal lung development.

**THERAPEUTIC RELEVANCE**

Specific targeting of pro-apoptotic pathways in the lungs has been shown to affect the outcome of acute lung injury in murine models. In vivo administration of caspase- and Fas inhibitors in adult mice attenuates LPS-induced acute lung injury, whereas Fas activating antibody causes increased lung permeability and histopathological alterations. Recent clinical and experimental observations have suggested that the renin-angiotensin system is involved in the pathogenesis of acute lung injury. A high activity of angiotensin converting enzyme (ACE), leading to an increase of angiotensin II, is believed to have a detrimental effect on the susceptibility and outcome of ARDS, whereas high ACE2 activity, leading to a decrease of angiotensin II, was found protective. Interestingly, it has been shown in vitro that Fas-mediated apoptosis of alveolar epithelial cells require angiotensin II binding to its cell receptor. Lukkarinen et al reported that angiotensin II receptor inhibition reduces epithelial cell apoptosis in ventilated surfactant-deficient rats. However, a relationship with ACE gene polymorphism and outcome of acute lung injury, as has been found for adults, could not be demonstrated in ventilated very low birth weight infants. Finally, animal studies have shown that both ACE and angiotensin II inhibition led to a decrease of meconium-induced lung injury and lung epithelial cell apoptosis.

The influence of corticosteroids on lung epithelial cell apoptosis is controversial. Dorscheid et al have reported an increase of primary airway epithelial cell apoptosis with induced cytochrome c release and caspase activity following continuous exposure to corticosteroids in vitro. In contrast, Wen et al showed reduced apoptosis of A549 cultured cells after treatment with a high concentration of dexamethasone. In line with these results, dexamethasone was found to significantly diminish bleomycine-induced acute lung injury, lung epithelial cell apoptosis and Fas/FasL expression in mice. To our knowledge, there are no data on the effects of corticosteroids on lung epithelium apoptosis specifically focusing on age differences.

Future animal and human experiments should reveal the clinical relevance of these studies in the search of new treatment strategies for both adult and pediatric patients with acute lung injury.
CONCLUSIONS

Accumulating evidence shows that dysregulation of apoptosis of lung epithelial cells is a key event in the pathogenesis of acute lung injury. Although research specifically focusing on children is limited, there is considerable evidence showing that this mechanism is also very relevant for pediatric acute lung injury. The consequences of dysregulation of apoptosis in infants and young children, in whom a tight regulation of apoptosis is essential for normal development and maturation, are incompletely understood. One could hypothesize that both short- and long term outcome of acute lung injury in children is influenced by altered susceptibility to pro- and anti-apoptotic events as part of their developmental stage. Additional research into this subject is therefore highly needed. Increased insight into the mechanisms that modulate apoptosis in acute lung injury may lead to novel therapeutic strategies. Several promising anchor points, including manipulation of the renin-angiotensin system, have already been identified. In addition, knowledge of the mechanisms that link apoptosis with cell stretch and hyperoxia may help identify better respiratory support strategies for critically ill children with acute lung injury.
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Active Caspase-3 in Lung Epithelium of Children with ARDS

Reinout A. Bem¹
Chris M. van der Loos²
Job B.M. van Woensel¹
Albert P. Bos¹

¹ Pediatric Intensive Care Unit, Emma Children’s Hospital; ² Department of Pathology, Academic Medical Center Amsterdam, The Netherlands

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ABSTRACT

The objective of this observational study was to investigate the extent of active caspase-3 immunostaining in lung epithelial cells in children with acute respiratory distress syndrome (ARDS). We performed double immunohistochemistry for cleaved caspase-3 and (pan) cytokeratin in lung tissues obtained at autopsy of sixteen children who died with ARDS and diffuse alveolar damage. Spectral imaging was used for the quantification of immunohistochemistry co-localization of these markers. We found a wide range in the percentage of alveolar epithelial cell surface area with positive active caspase-3 staining in the lungs of children with ARDS (from 1 up to almost 20%). The degree of caspase-3 immunostaining in epithelial cells positively correlated with age. We conclude that there is a high variability in the extent of classical apoptosis in lung epithelial cells in pediatric ARDS, potentially in part dependent on age.
INTRODUCTION

Acute respiratory distress syndrome (ARDS) is a major cause of death among intensive care patients worldwide. ARDS is associated with marked (neutrophilic) alveolitis and enhanced lung permeability due to endothelial and epithelial cell dysfunction. An important mechanism of epithelial injury in ARDS is the activation of specific pro-apoptotic pathways in lung epithelial cells. Patients who died with ARDS have increased markers of apoptosis, such as cleaved caspase-3, in cells in the alveolar walls. Furthermore, several animal studies modeling both indirect and direct causes of ARDS have shown that enhanced lung cell apoptosis is associated with leakage of serum proteins into the lungs and histopathological alterations, which may be attenuated by inhibiting pro-apoptotic pathways.

So far, studies investigating classical apoptosis by active caspase-3 in lung tissues of ARDS patients have not included specific immunostainings of lung cells, and thus remain limited in the interpretation of cellular distribution of this marker. The purpose of the present brief report was to study the extent of active caspase-3 immunolocalization specifically in lung epithelial cells in pediatric ARDS. To this aim, we performed double immunohistochemistry for active caspase-3 and (pan)cytokeratin in lung tissue sections of sixteen children who died with ARDS and diffuse alveolar damage. Visual detection of co-localizing double staining by light microscopy by eye can be compromised by the loss of color contrast, especially in diffuse injured tissues. Therefore, to quantify co-localization we used the technique of spectral imaging, which can unmix multicolor microscopy into individual images based on the detection of their spectral characteristics.

METHODS

Patients

Formalin-fixed, paraffin-embedded lung tissue samples from autopsy of 16 mechanically ventilated patients with ARDS who died after admission to the Pediatric Intensive Care Unit (PICU) in the period 1990-2009 were obtained from the Department of Pathology of the Academic Medical Center Amsterdam and the University Medical Center Utrecht, The Netherlands. All selected patients met the American European Consensus Conference criteria of ARDS. The lung tissue cubes were obtained by standard hospital autopsy procedures and fixed in buffered formalin for 24 hr. The need for written informed consent was waived in consultation with the medical ethical research committees in both medical centers.

Immunohistochemistry

Of each lung tissue sample 5-μm-thick sections were cut for double-staining for active caspase-3 and cytokeratin. Sections were xylene-deparaffinized, rehydrated in an alcohol
series, and treated for 20 minutes with 0.3% H$_2$O$_2$ in methanol to block endogenous peroxidase activity. Heat induced antigen retrieval was performed in Tris-EDTA (pH 9.0) at 98°C. After washing in tap water, the sections were treated with Ultra V Block (Immunologic, Duiven, The Netherlands) to block non-specific binding sites. Primary antibody labeling was performed with a cocktail of rabbit anti-human cleaved caspase-3 (Asp175) mAb (Cell Signaling, Danvers, MA) and mouse anti-human cytokeratin 1-8, 10, 13-16 and 19 mAb (AE1/AE3 clone, Dako, Glostrup, Denmark), followed by secondary labeling with a cocktail of anti-rabbit IgG HRP polymer and anti-mouse AP polymer (Immunologic). Visualization of AP activity was performed with AP Substrate Kit (Vector$^\text{®}$Blue, Vector Laboratories, Burlingame, CA) and HRP activity was detected with Peroxidase Substrate Kit (Vector$^\text{®}$NovaRED, Vector Laboratories). The sections of all patients were stained in the same experiment.

Quantification
Both the cleaved caspase-3 and cytokeratin staining have cytoplasmic localization. Detection of co-localization of the Vector$^\text{®}$NovaRED and Vector$^\text{®}$Blue signals was performed by spectral imaging, as described previously in detail$^6$. Per section 5 random digital images containing alveolar structures at moderate (200x) magnification were taken by a Leica$^\text{®}$ microscope using Nuance 2.8.0. software (CRi; Woburn, MA). After loading the spectral characteristics containing the individual spectra of Vector$^\text{®}$NovaRED and Vector$^\text{®}$Blue, the Nuance software was used to unmix the spectra in these images. The spectrum of erythrocytes and non-specific staining was filtered out to avoid overlap. For quantification the co-localization tool of the Nuance 2.8.0. software was used to calculate the percentage of pixels with positive cytokeratin signal co-localizing with positive cleaved caspase-3 signal per image.

The same threshold and spectral settings were used for analysis in all images. Observer agreement of signal co-localizations was checked in all images. In ten random 200x images we visually counted the number of double positive cells to assess the degree of correlation with the spectral imaging quantification method.

Statistical analysis
Not normally distributed data are shown as box plots including median, interquartile range and full range, and were analyzed by using the Mann-Whitney U test for differences between groups. Spearman’s $r$ was calculated to assess correlation.

RESULTS

Patient characteristics are presented in Table 1. Cause of death was multiple organ dysfunction (n=9, including central nervous system failure: n=4), respiratory failure (n=6) or circulatory failure (n=1). All patients had been treated with supplemental oxygen and mechanical ventilation. Two patients had been treated with high frequency oscillatory ventilation...
Table 1. Patient characteristics (n=16).

| Age; year (SE) | 7.9 (1.6) |
| Male; number of patients (%) | 9 (56) |
| Duration of mechanical ventilation; days (SE) | 9 (2.3) |
| Clinical disorders; number of patients (%) |
| Direct: pneumonia |
| infectious | 4 (25) |
| aspiration | 4 (25) |
| other/unknown | 4 (25) |
| Indirect: sepsis | 4 (25) |
| Underlying condition; number of patients (%) |
| (Hemato-)oncology | 9 (56) |
| (Auto-)immunology | 3 (19) |
| Immunosuppression | 5 (31) |
| leucopenia1 | 2 (13) |

1 WBC < 500 x 10^6 / L. SE: standard error of mean.

Figure 1. A-C, lung tissue sections of ARDS patients stained with haematoxylin and eosin (magnification 100x) showing changes characteristic of exudative ARDS (*protein-rich alveolar exudates) (A), hyaline membranes (arrow) (B), and marked fibroproliferative changes (C). D-F, Immunohistochemistry for cleaved (active) caspase-3 (magnification 500x) showing the wide range of positive staining in lung tissues sections of patients with ARDS: from widespread positive cells in the alveolar walls (arrows) (D), to almost no positive cells in the alveolar walls (E). Note that the latter picture is comparable to the finding in lung tissue of a patient who died with sepsis, but with normal lung architecture and without clinical ARDS (F).

at some point during admission. The lung histopathology of the patients showed diffuse alveolar damage, with alterations such as (neutrophilic) alveolitis and evidence of endothelial/epithelial dysfunction (e.g. capillary congestion, intra-alveolar protein-rich edema and protein
precipitation with hyaline membranes, hemorrhage). Alterations indicating early exudative phase of ARDS were seen in only two patients (Figure 1A); the remaining fourteen patients showed evidence of early to late proliferative and fibrotic changes (Figure 1B-C).

Interestingly, the degree of active caspase-3 immunostaining in the lung tissues was highly variable between the ARDS patients, ranging from scarce positive lung cells to widespread staining in alveolar wall cells (Figure 1D-E). This finding was not related to inhomogeneous distribution of pathological changes, as we found comparable results in individual children of whom sections from multiple sites/lobes had been obtained. In injured lungs with extensive cellular infiltrates it may be difficult to reliably determine the cellular distribution of caspase-3 immunostaining. For this reason, we next performed double immunohistochemistry including an epithelial specific marker, (pan)cytokeratin. In addition, we used spectral imaging for quantification of the degree of co-localization of the signals (Figure 2A-B). Again, by this method there was a high variability between the

Figure 2. A, representative composite images of cytokeratin (blue) and active caspase-3 (red) double staining of lung tissue after unmixing of the individual color spectra, showing co-localization (yellow) (zoomed from 200x magnification images); B, original RedGreenBlue-bright field images (upper left) and composite images: without highlighted co-localization (upper right), active caspase-3 images (lower left) and cytokeratin images (lower right); C, percentage of cytokeratin (ck) positive staining co-localized with active caspase-3 staining in alveolar tissue sections of ARDS patients with indirect lung injury (n=4) or direct lung injury (n=12); D, correlation between the percentage of cytokeratin (ck) positive staining co-localized with active caspase-3 staining and age (r=0.5, p<0.05).
patients with ARDS: the median (range) percentage of positive cytokeratin pixels (surface area) co-localizing with positive active caspase-3 signal per image was 6.1% (1.0-18.1%). Importantly, we found a high correlation between visually counted double signal positive cells counted and the spectral imaging quantification ($r=0.9$, $p<0.01$, not shown).

No clear association was found between the percentage of cytokeratin staining co-localizing with active caspase-3 staining and cause of lung injury (indirect versus direct) (Figure 2C) or duration of mechanical ventilation ($r=0.3$, $p=ns$, not shown). For ARDS pathological stage a potential association could not be determined because almost all patients showed progressed alveolar injury. Interestingly, in our cohort, age was positively correlated with the degree of cytokeratin/active caspase-3 double positive staining (Figure 2D).

**DISCUSSION**

Classical (caspase-dependent) apoptosis is considered an important mechanism of epithelial injury in ARDS\(^1\). In the present observational report we studied active caspase-3 immunostaining specifically in epithelial cells in lung tissues of children who died with ARDS. We introduce spectral imaging to quantify the extent of co-localization of active caspase-3 and the epithelial marker (pan)cytokeratin.

Our methods have the following advantages. First, the double immunostaining increases our insight into caspase activation specifically in lung epithelial cells, in this case alveolar epithelial cells as images contained alveolar structures only. Second, the use of spectral imaging overcomes problems with loss of color contrast often encountered in double immunostainings with co-localizing signals\(^6\), and it quantifies the findings fast and likely more accurate over a large tissue area in comparison to semi-quantified data from visual counting by light microscopy. Finally, in the current analysis, spectral imaging helps to determine how much of the lung epithelium actually is involved in terms of activation of caspase-3; it can be used to calculate the percentage of epithelial surface area that shows active caspase-3 staining. Importantly, there was a high correlation between these quantified data based on double positive surface area (pixels) and the number of epithelial cells deemed positive for caspase-3 as visually counted.

The main finding of this study is a high variability in active caspase-3 staining in lung epithelium between the children with ARDS. We found a range including patients with almost no caspase-3 positive epithelial cells, comparable to a patient without ARDS, to patients with widespread positive caspase-3 staining in up to almost 20% of the epithelial surface area. Evidently, a number of different factors may have contributed to this variability. First, immunohistochemical staining of autopsy samples is limited to ‘snapshot’ analysis, and does not appreciate the dynamic character of apoptosis. Second, as is true in general in the ARDS population, the patients in our study were highly heterogeneous.
with respect to clinical disorders and underlying illnesses and their treatments. In this light, our study once more highlights the challenge encountered in ARDS research due to the non-specific clinical criteria on which the diagnosis is based. Although we found no association between the extent of caspase-3 activation and the above mentioned factors, we have to carefully interpret these findings because of the retrospective design and small number of patients in this study. Interestingly, despite this limitation, age positively correlated with the extent of caspase-3 immunostaining in lung epithelial cells. This finding is in line with emerging experimental and clinical studies that suggest age and lung development are important factors influencing pro-apoptotic pathways during lung injury, as well as the susceptibility and outcome of ARDS.\textsuperscript{8-13}
REFERENCES


Depletion of Resident Alveolar Macrophages Does Not Prevent Fas-mediated Lung Injury in Mice

Reinout A. Bem, Alex W. Farnand, Venus Wong, Amy Koski, Michael E. Rosenfeld, Nico van Rooijen, Charles W. Frevert, Tom R. Martin, Gustavo Matute-Bello

1 Research Service of the VA Puget Sound Health Care System and 2 Center for Lung Biology, Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of Washington, Seattle (WA); 3 Department of Pathobiology, Department of Medicine, University of Washington, Seattle (WA); 4 Department of Cell Biology, Faculty of Medicine, Free University, Amsterdam, The Netherlands

ABSTRACT

Activation of the Fas/FasL system in the lungs results in a form of injury characterized by alveolar epithelial apoptosis and neutrophilic inflammation. Studies in vitro show that Fas activation induces apoptosis in alveolar epithelial cells and cytokine production in alveolar macrophages. The main goal of this study was to determine the contribution of alveolar macrophages to Fas-induced lung inflammation in mice, by depleting alveolar macrophages using clodronate-containing liposomes. Liposomes containing clodronate or PBS were instilled by intratracheal instillation. After 24 hr, the mice received intratracheal instillations of the Fas-activating monoclonal antibody Jo2 or an isotype control antibody, and were studied 18 hours later. The Jo2 mAb induced increases in broncholalveolar lavage fluid (BALF) total neutrophils, lung caspase-3 activity, BALF total protein, and worsened histological lung injury in the macrophage-depleted mice. Studies in vitro showed that Fas activation induced the release of the cytokine KC in a mouse lung epithelial cell line, MLE-12. These results suggest that the lung inflammatory response to Fas activation is not primarily dependent on resident alveolar macrophages and may instead depend on cytokine release by alveolar epithelial cells.
INTRODUCTION

Acute lung injury (ALI) and its more severe form, the acute respiratory distress syndrome (ARDS) are acute-onset inflammatory lung conditions characterized by hypoxemia and bilateral lung infiltrates, in the setting of a normal cardiac preload. Each year, more than 175,000 patients develop ALI/ARDS in the United States, and of these approximately 74,000 die. However, the initial pathogenic events leading to ALI/ARDS remain unclear, and treatment is limited to supportive measures.

The receptor-ligand system Fas/FasL has been implicated in the pathogenesis of ALI in humans and animals. Fas (CD95) is a cell surface receptor which in the lungs is expressed in airway and alveolar epithelial cells, in neutrophils, and in alveolar macrophages. The natural ligand of Fas, FasL (CD178) exists in a soluble form and in a membrane-bound form. In the lungs, the membrane-bound form of FasL is expressed in the airway and alveolar epithelia, as well as in resident and migrating leukocytes. The soluble form of FasL (sFasL) results primarily from proteolytic cleavage of membrane-bound FasL by MMP-7 (matrilysin), although it can also be released by activated monocytes. Initial studies had suggested that sFasL was primarily a negative regulator of membrane FasL, but subsequent studies showed that sFasL retains its bioactivity in the lungs, both in vivo and in vitro.

The Fas/FasL system has been traditionally considered a prototypical pro-apoptotic system. Binding of Fas to FasL triggers activation of a series of cysteine proteases collectively known as caspases, which eventually leads to apoptosis. However, studies performed in vitro have established that binding of Fas to FasL can also lead to activation of pro-inflammatory pathways, activation of the nuclear factor κB, and release of pro-inflammatory cytokines, including the neutrophil chemoattractant IL-8 (CXCL8). The role of the pro-apoptotic function of the Fas/FasL system in human and experimental lung injury has been extensively studied (reviewed in), but the role of the pro-inflammatory function of Fas in the pathogenesis of lung injury remains less clear.

Earlier studies investigating activation of the Fas/FasL system in the lungs consistently found evidence of an inflammatory response characterized by cytokine release and neutrophil recruitment into the lungs. The relevance of the pro-inflammatory function of the Fas/FasL system was confirmed in studies demonstrating that mice deficient in Fas have an impaired neutrophilic response to inhaled LPS, and that silencing of Fas in the lungs protected against lung inflammation in a model of hemorrhagic shock followed by cecal ligation and puncture. Furthermore, blockade of the Fas/FasL system by specific pharmacologic inhibitors or by the lpr mutation resulted in reduced BALF neutrophil counts and lower concentrations of TNF-α and MIP-2 48 hr after intratracheal instillation of S. pneumonieae. Taking together, these studies suggest that the Fas/FasL system may play an important role not just in apoptosis, but also in the development of inflammation.
of an inflammatory response in the lungs following exposure to LPS, live bacteria, and sepsis.

An important question is whether the inflammatory response associated with the Fas/FasL system results from a direct pro-inflammatory effect of Fas signaling in specific lung cells, or instead is secondary to an initial apoptotic injury in the lungs. Studies by Park et al. showed that human macrophages incubated with human recombinant sFasL or the agonistic antibody CH-11 in vitro do not become apoptotic, but instead release pro-inflammatory cytokines such as TNF-α and IL-8. Interestingly, in the Park study macrophages released similar amounts of IL-8 in response to 500 ng/mL of sFasL and to 1 µg/mL of LPS. In contrast, the responses of alveolar epithelial cells to FasL in vitro include both apoptosis and release of IL-8. These in vitro studies led to the initial hypothesis that Fas-induced lung injury resulted from a combination of pro-inflammatory responses in macrophages, leading to cytokine release and neutrophil migration, and alveolar epithelial apoptosis, leading to disruption of the epithelial barrier. This hypothesis was tested in vivo using chimeric mice lacking Fas in either myeloid or non-myeloid cells, and the prediction was that following Fas activation, the mice expressing Fas in macrophages would develop an inflammatory response, and the mice expressing Fas in their epithelium would develop alveolar epithelial apoptosis and enhanced lung permeability. However, the prediction was wrong; the mice expressing Fas only in their myeloid cells showed little response to Fas activation, while the mice expressing Fas in their epithelium showed evidence of both inflammation and apoptosis, suggesting that the inflammatory response to Fas in the lungs was independent of Fas activation in macrophages. It is possible that in the chimera study, resident alveolar macrophages might have been activated in response to exposure of the basement membrane resulting from apoptosis of alveolar epithelial cells, or alternatively in response to phagocytosis of apoptotic epithelial cells. Therefore, the question of whether macrophages were responsible for cytokine production and inflammation following Fas activation remained unclear.

The goal of the present study was to determine whether resident alveolar macrophages are required for the development of Fas-induced lung inflammation in mice, using a model of clodronate depletion of lung alveolar macrophages. Furthermore, we investigate whether murine alveolar epithelial cells release cytokines in response to Fas activation. The main findings are that macrophage-depleted mice developed a neutrophilic inflammatory response following Fas activation with the Fas-activating antibody Jo2 in vivo, and that the murine alveolar epithelial cell line MLE-12 releases the neutrophil chemoattractant KC in response to Fas activation in vitro.
METHODS

Reagents
Clodronate (clod, dichloromethylene diphosphonate)-encapsulated liposomes and phosphate buffered saline (PBS)-encapsulated liposomes were prepared as described before [27]. Clodronate was a kind gift of Roche diagnostics GmbH, Mannheim, Germany. The liposomes were stored up to 2 weeks at 4°C in sealed tubes containing N₂. Purified hamster anti-mouse Fas mAb Jo2, LPS-free, azide free, was purchased from BD PharMingen (San Diego, CA). Purified hamster anti-keyhole limpet hemocyanin IgG₂, also from BD PharMingen, was used as isotype control mAb. Antibodies used for immunohistochemistry included rat anti-mouse mac-2 (Accurate Laboratory Research Products, Westbury, NY), rat anti-F4/80 mAb (Serotec Inc, Raleigh, NC), rabbit anti-CX₃CR1 Ab (Anaspec, San Jose, CA), goat anti-CCR2 Ab (Abcam, Camebridge, UK), biotinylated mouse-anti rat IgG, chicken anti-rat IgG-Alexa Fluor 647, chicken anti-rabbit IgG- Alexa Fluor 488 and donkey anti-goat IgG-Alexa Fluor 546 (all Zymed, Invitrogen, Carlsbad, CA).

Animal protocols
The animal protocols were approved by the Animal Care Committee of the VA Puget Sound Healthcare System, Seattle, WA. Briefly, male C57BL/6 mice weighing 25-30 g (Jackson Laboratories, Bar Harbor, ME) were anesthetized with inhaled isoflurane and placed on an inclined surface. The larynx was visualized and the trachea was intubated with a gavage tube attached to a 1.0 ml syringe containing 100 µl of water. Intubation of the trachea was verified by movement of the water meniscus in the syringe during the animal’s respiratory efforts. After endotracheal intubation, each mouse received 100 µL of liposomes in a single aliquot through the endotracheal tube. The tube was removed and the mice were allowed to recover from anesthesia and return to their cages with free access to food and water.

The mice were re-anesthetized and re-intubated 24 hr after instillation of the liposomes, and received instillations of Jo2 mAb or an isotype control IgG, 2.5 µg/g. After the instillations, the mice were allowed to recover from anesthesia and returned to their cages with free access to food and water. Eighteen hours later, the mice were euthanized with an intraperitoneal injection of pentobarbital (120 mg/kg) and exsanguinated by closed intracardiac puncture. The thorax was opened and the trachea cannulated and secured. The left hilum was clamped and the left lung was removed and placed in a tube containing sterile H₂O plus protease inhibitor (Roche Applied Science, Indianapolis, IN) for homogenization. After removing the left lung, the right lung was lavaged with a 0.6 ml aliquot of 0.9 % NaCl containing 0.6 mM EDTA, followed by three separate 0.5 ml aliquots. The BAL aliquots were pooled for further analysis. Immediately after the BAL procedure, the right lung was fixed with 4% paraformaldehyde at 15 cm H₂O for histological analysis.
Experimental design
First, to determine the extent and duration of alveolar macrophage depletion induced by clodronate, mice were treated with intratracheal PBS- or clodronate liposomes and then studied after 24, 48 or 72 hr (n=3/group per time).

In a second set of experiments, mice were assigned to one of four groups: PBS-liposomes + control IgG mAb (PBS + IgG, n=6); clodronate liposomes + control IgG mAb (Clod + IgG, n=6); PBS-liposomes + Jo2 mAb (PBS + Jo2, n=6); and clod-liposomes + Jo2 mAb (Clod + Jo2, n=6).

Sample processing
The BALF aliquots from each mouse were pooled, and an aliquot was processed immediately for total cell counts and differentials. The remainder of the BALF was spun at 200 x g and the supernatants were stored in individual aliquots at -70°C. Each left lung was homogenized in 1.0 ml of sterile H2O with protease inhibitors (Roche Diagnostics Corporation, Indianapolis IN). The lung homogenate was divided into aliquots for later cytokine and myeloperoxidase (MPO) measurements. For cytokine and caspase-3 activity measurements, an aliquot of the lung homogenate was vigorously mixed with a buffer containing 0.5% Triton-X-100, 150 mM NaCl, 15 mM Tris, 1 mM CaCl2 and 1mM MgCl2, pH 7.40, incubated for 30 min at 4°C, and then spun at 10,000 x g for 20 min. The supernatants were stored at -70°C. For MPO measurements, the homogenate was vigorously mixed with 50 mM potassium phosphate, pH 6.0, with 5% hexadecyltrimethyl ammonium bromide (Sigma-Aldrich, St. Louis, MO) and 5 mM EDTA. The mixture was sonicated and spun at 12,000 x g for 15 min at 25°C, and the supernatant was stored at -70°C.

Measurements
Total cell counts and differentials. Total cell counts were performed on an aliquot of the BALF, using a hemacytometer. Differential cell counts were performed on cytospin preparations stained with the Diff-quick method (Andwin Scientific, Addison IL).

Myeloperoxidase was measured in lung homogenates using the Amplex Red fluorometric assay, following instructions from the manufacturer (Molecular Probes, Eugene, OR).

Permeability measurements. The total protein concentration in BALF was measured using the bicinchoninic acid method (BCA assay, Pierce Co., Rockford, IL). The concentration of IgM in BALF was measured with a specific mouse immunoassay (R&D Systems, Minneapolis MN). After dilution of the samples, the lower limit of detection of the IgM assay was 20 ng/mL.

Cytokine measurements. The cytokines TNF-α, IL-1β, MIP-2, KC, GM-CSF, VEGF, IFN-γ and IL-6 were measured in lung homogenates using the Fluorokine MultiAnalyte Profiling kits (R&D systems, Minneapolis, MN) for a multiplex fluorescent bead assay (Luminex, Austin, TX). After dilution of the samples, the lower limits of detection were 18.7 pg/mL for TNF-α, 130.1 pg/mL for IL-1β, 29.1 pg/mL for KC, 21.1 pg/mL for MIP-2, 23.1 pg/mL for GM-CSF, 13.2 pg/mL for VEGF, 63.1 pg/ml for IFNγ and 27.2 pg/mL for IL-6.
**Caspase-3 activity** in lung homogenates was measured with the caspase-3/CPP32 Fluorometric Assay kit (Biovision Inc., Mountain View, CA). Lung homogenate aliquots were diluted 1:2 in assay reaction buffer containing 10mM DTT and incubated for 2 hr at 37°C with the caspase-3 specific substrate DEDV-AFC (50µM). Fluorescence was read with a fluorometer using 400 nm excitation and 505-nm detection filters. Results are shown as arbitrary fluorescence units.

**Histopathology and immunohistochemistry**

The sections were deparaffinized by heating to 57°C for 60 min and rehydrated by washing twice in xylene for 5 min, twice in 100% ethanol for 3 min, twice in 95% ethanol for 3 min, and once in dH₂O for 5 min. The slides were then washed three times in PBS for 5 min and treated with 0.3% Triton X-100 for 30 min at room temperature. After washing in PBS three times for 5 min, endogenous peroxidases were blocked with Peroxo-Block (Zymed, Invitrogen, Carlsbad, CA) for 90 seconds at room temperature. Next, the slides were washed again in PBS, treated with boiling 10 mM citric acid, 0.05% Tween-20, pH 6.0 for 15 min and blocked 30 min at room temperature with Dako Serum-Free Protein Block (Dako, Carpinteria, CA). The tissues were then labelled with rat anti-mac-2 mAb overnight at 4°C in a moist chamber. After washing in PBS for three times, the tissues were labelled with biotinylated mouse anti-rat mAb for 30 min at room temperature for mac-2 and washed in PBS three times. The slides were then labelled with streptavidin horseradish peroxidase conjugate (Zymed) for 10 min at room temperature, rinsed three times with PBS, and developed with AEC Peroxidase Substrate (Zymed) for 7.5 min. The slides were then rinsed with running deionized H₂O for 5 min, counterstained with 1% methyl green for 6 min and mounted with glycerol-vinyl-alcohol (Zymed).

For triple labeling for F4/80, CX₃CR1 and CCR2 the slides were blocked with Dako Serum-Free Protein Block containing 3% goat serum and donkey serum (Jackson Immunoresearch, West Grove, PA). The slides were then labelled with the rat anti-F4/80 mAb (Serotec Inc, Raleigh, NC), rabbit anti-CX₃CR1 Ab (Anaspec, San Jose, CA) and goat anti-CCR2 Ab (Abcam, Cambridge, UK) for 1 hr at room temperature, and with the secondary antibodies (chicken anti-rat IgG-Alexa Fluor 647, chicken anti-rabbit IgG- Alexa Fluor 488 and donkey anti-goat IgG-Alexa Fluor 546 (all Zymed, Invitrogen)) for 40 min at room temperature. For detection, the slides were treated with Sudan Black (Fisher, Pittsburgh, PA), rinsed with running deionized H₂O for 5 min and mounted with ProLong Gold Anti-gade reagent (Zymed, Invitrogen). The fluorescence signal was visualized using a confocal microscope (LSM510, Carl Zeiss Inc, Thornwood, NY). For quantification, we counted the number of positive cells in 5 randomly selected high power fields (400x) per tissue section.

DNA nick-end labeling assays (TUNEL) (TACS in situ Apoptosis Detection Kit; Trevigen Inc., Gaithersburg, MD) were performed as previously described. For quantification of
the TUNEL assay, we counted the number of positive cells in each of the 12 randomly generated fields per tissue section, at a magnification of 400X.

**Cellular studies**

MLE-12 mouse lung epithelial cells (ATCC No. CRL-2210) were cultured at 37°C, 5% CO\(_2\) in DMEM/F12 (with Ham formulation) (Invitrogen, Carlsbad, CA) supplemented with 2% fetal bovine serum (Hyclone, Logan, UT) 1% penicillin/streptomycin (Invitrogen), 1% L-Glutamine (Invitrogen), 1% HEPES (Sigma-Aldrich, St. Louis, MO), 1% Insulin/Transferrin/Sodium Selenite (Invitrogen), 0.01% β-Estradiol (Sigma-Aldrich) and 0.01% Hydrocortisone (Sigma-Aldrich). MLE-12 cells are SV40-transformed mouse lung epithelial cells that show several features of type II cells, including the presence of microvilli, intracellular multilamellar inclusion bodies in the cytoplasm, and expression of the surfactant proteins B and C. The cells were seeded in 96 well tissue-culture plates (Costar, Cambridge, MA) and incubated at 37°C, 5% CO\(_2\) until reaching 70-80% confluence, at which point the media was replaced with fresh media supplemented with serial concentrations of either Jo2 mAb or an isotype control IgG, with or without the broad caspase-inhibitor zVAD-fmk (100 µM) (Axxora, San Diego, CA). After 18 h, the supernatants were collected for measurement of KC concentration by ELISA (R&D systems, Minneapolis, MN), and cell survival was measured using alamar Blue (BioSource, Camarillo, CA) as described previously.

**Statistical analysis**

Comparisons between multiple groups were performed using one-way ANOVA. Significance between groups was determined with the Fisher’s Least Significant Difference (LSD) post-hoc test. A p value of <0.05 was considered statistically significant. Data are reported in the text as means ± SEM, and shown in figures as box and whisker plots depicting individual data points and the median, the interquartile ranges and the 10\(^{th}\) and 90\(^{th}\) percentiles. The data in figures 5 and 8 are shown as means ± SEM.

**RESULTS**

**Clodronate Liposomes induce macrophage depletion in mice**

To determine the extent of macrophage depletion induced by clodronate we administered intratracheal liposomes containing PBS or clodronate to normal mice, and then performed cell counts and differentials in BALF recovered at 24, 48 or 72 hr after liposome instillation (n = 3/group). Macrophage depletion was maximal 24 hr after treatment with clodronate liposomes, and the decrease in total alveolar macrophages was approximately one order of magnitude as compared to the mice treated with PBS-liposomes (0.2 ± 0.1 x 10\(^5\) vs 2.7 ± 0.3 x 10\(^5\) cells, respectively) (Figure 1A). The clodronate liposomes did induce a small degree
of neutrophil recruitment, in the order of $10^3$ total cells, that persisted for 72 hr (Figure 1B). Lipopolysaccharide was not detected in the clodronate- or PBS-liposomes using the Limulus amebocyte assay.

To determine whether the neutrophil response seen in the BALF of the clodronate-liposome treated mice was associated with tissue damage, we examined lung tissue sections from mice euthanized 24 hr after treatment with liposomes containing PBS- (Figure 1C) or clodronate (Figure 1D). These sections confirmed that clodronate liposomes induced macrophage depletion. Importantly, no neutrophilic infiltrates were seen in the alveolar spaces or the interstitium of the mice treated with clodronate liposomes, and the normal lung architecture was preserved.

Based on these data, in the remaining experiments we administered In either a control IgG mAb or Jo2 mAb at 24 hr after liposome instillation. The mice were euthanized and

**Figure 1.** Total alveolar macrophages (A) and PMN (B) counts in the BALF of mice 24, 48 or 72 hr after intratracheal instillation of liposomes containing PBS (white) or clodronate (gray) (n=3/group for each time). Panel C and D show representative lung tissue sections from mice studied 24 hr after intratracheal instillation of liposomes containing PBS or clodronate respectively (H&E staining, magnification 400x). Note the absence of macrophages and neutrophils in the lungs from the mouse treated with clodronate liposomes (D).
studied 18 hours after administration of the antibodies. One animal in the PBS + IgG group died after PBS-liposome instillation.

The lung neutrophilic response to Jo2 mAb is not impaired by macrophage depletion

After treatment with clodronate liposomes, the BALF macrophage count decreased to 5.1 ± 1.1 x 10^4 cells in the mice receiving the nonspecific IgG (p < 0.05 compared to the PBS + IgG and the PBS + Jo2 groups), and to 7.7 ± 1.8 x 10^4 cells in the mice instilled with Jo2 mAb (p < 0.05 compared to the PBS + Jo2 group) (Figure 2A). Despite having a lower macrophage count, the mice treated with clodronate liposomes and Jo2 had a total BALF PMN count of 25.8 ± 4.4 x 10^4 cells, which was significantly increased as compared to each of the other treatment groups (0.06 ± 0.06 x 10^4 cells in the mice treated with PBS + IgG; 2.6 ± 1.6 x 10^4 cells in the mice treated with PBS + Jo2; and 6.0 ± 3.1 x 10^4 cells in the mice treated with clodronate + IgG, Figure 2B).

As an additional measurement of the neutrophil response, we assessed the total neutrophilic content in the lungs by measuring MPO activity in whole lung homogenates. Whole lung MPO activity was significantly increased in the mice treated with clodronate

![Figure 2.](image-url)

Figure 2. Total BALF macrophage (A), PMN (B) and lymphocyte (C) counts and lung homogenate MPO activity (D) in mice treated with intratracheal liposomes containing PBS or clodronate followed 24 hr later by intratracheal instillations of Jo2 mAb or control IgG mAb, 2.5 µg/g, and studied 18 hr later. n=6/group except for the PBS + IgG group, n=5.
liposomes and Jo2, as compared to the mice treated with clodronate liposomes and IgG (0.8 ± 0.1 vs 0.3 ± 0.0 cells, p < 0.05), but was similar to that of mice treated with PBS-liposomes and Jo2 (Figure 2D). Thus, the combined BALF and MPO data suggest that macrophage depletion did not prevent the neutrophilic response to Jo2, and may have even enhanced it.

The Jo2 mAb also induced a lymphocytic response in the BALF, which was highest in the mice treated with clodronate liposomes (Figure 2C).

The lung cytokine response to Jo2 mAb is not impaired by macrophage depletion
The administration of Jo2 mAb was associated with a trend towards an increase in all of the cytokines tested, and in almost all cases this increase was independent of macrophage depletion (Figure 3). These findings suggest that the cytokines tested did not originate in resident alveolar macrophages.

Clodronate treatment did not prevent Fas-mediated histologic lung injury
The mice in the PBS + IgG group exhibited normal lung histology (Figure 4A). The administration of Jo2 mAb to mice treated with PBS-liposomes resulted in focal areas of inflammatory infiltrates (Figure 4B). The lungs from mice treated with clodronate liposomes and IgG appeared normal, except for the absence of AM (Figure 4C). In contrast, the administration of Jo2 mAb to mice treated with clodronate liposomes was followed by inflammatory infiltrates and alveolar wall thickening (Figure 4D). Thus, the administration of Jo2 resulted in histologic lung injury regardless of the presence or absence of resident macrophages.

To confirm the extent of macrophage depletion induced by clodronate, we performed immunohistochemistry using the monocyte/macrophage marker Mac-2 (Figure 4, E-H). Mac-2 recognizes galectin-3, a β-galactoside-binding lectin which is highly expressed in macrophages. The lungs of mice treated with PBS-liposomes contained intra-alveolar cells staining for Mac-2 (arrows) (Figure 4, E-F). In contrast, the lungs from mice treated with clodronate liposomes and control IgG showed very few cells staining for Mac-2 (Figure 4H). However, the mice treated with clodronate liposomes and Jo2 showed a small number of cells staining for Mac-2, suggesting the presence of either residual resident macrophages or newly recruited macrophages (Figure 4D). Alternatively, Mac-2 may be expressed in type II cells in areas of severe inflammation. To further confirm the extent of macrophage depletion, we performed additional immunohistochemistry using F4/80 mAb, which recognizes a 160 kD glycoprotein expressed by murine monocytes/macrophages. Clodronate-liposome administration resulted in a decrease in cells staining for F4/80, and this decrease was similar in the mice treated with IgG and Jo2 mAb (Figure 5A).
Figure 3. Cytokine concentrations in lung homogenates from mice treated with intratracheal liposomes containing PBS or clodronate, followed 24 hr later by intratracheal instillations of Jo2 mAb or control IgG mAb, 2.5 µg/g, and studied 18 hr later. The cytokines were measured simultaneously using a multiplex assay, and include: TNF-α (A), IL1-β (B), MIP-2 (C), KC (D), GM-CSF (E), VEGF (F), IFN-γ (G) and IL-6 (H). n=6/group, except for the PBS + IgG group, n=5.
Figure 4. Lung tissue sections from mice treated with intratracheal liposomes containing PBS or clodronate, followed 24 hr later by intratracheal instillations of Jo2 mAb or control IgG mAb, 2.5 µg/g, and studied 18 hr later. The right column shows representative lung tissue sections stained with H&E (400x, insets 200x). The left column shows immunohistochemistry for AM (arrows), using anti mac-2 antibody.
Resident alveolar macrophages are thought to express low levels of the fractalkine receptor, CX3CR1, and variable levels of the MCP-1 receptor, CCR2. Newly recruited, highly inflammatory monocytes show high expression of CCR2, but low expression of CX3CR1. Therefore, we investigated the expression of CX3CR1 and CCR2 in cells expressing F4/80. In all mouse groups, the majority of cells expressing F4/80 co-expressed CX3CR1 and CCR2 (Figure 5B). We found no evidence for an increase in F4/80+, CX3CR1-, CCR2+ cells, which have been associated with increased inflammation and extensive recruitment to inflammatory sites. Thus, the data suggest that the lung injury

![Figure 5](image_url)

**Figure 5.** Number of cells staining for F4/80 in lung tissue sections from mice treated with intratracheal liposomes containing PBS or clodronate, followed 24 hr later by intratracheal instillations of Jo2 mAb or control IgG mAb, 2.5 µg/g, and studied 18 hr later (A). Panel B shows the percentage of F4/80 cells co-staining for CX3CR1 or CCR2 in the same sections. Data is shown as means ± SEM. * p < 0.05 as compared to percentages of CX3CR1 or CCR2 positive cells.

![Figure 6](image_url)

**Figure 6.** Lung homogenate caspase-3 activity (arbitrary fluorescence units) (A) or TUNEL positive cells per 12 high-power fields (B) in mice treated with intratracheal liposomes containing PBS or clodronate, followed 24 hr later by intratracheal instillations of Jo2 mAb or control IgG mAb, 2.5 µg/g and studied 18 hr later. n=6/group, except for PBS + IgG, n=5.
seen in the mice treated with clodronate liposomes and Jo2 did not result from increased recruitment of CX3CR1-, CCR2+ monocytes, and supports the idea that the inflammatory response caused by Jo2 was driven by activation of cells other than macrophages.

**Apoptotic response to Fas activation in the setting of macrophage depletion**

As mentioned in the introduction, activation of the Fas/FasL system can lead to inflammation and also apoptosis. To determine the extent of apoptosis in the lungs we measured caspase-3 activity in whole lung homogenates. Caspase-3 activity was highest in the mice treated with clodronate liposomes and Jo2 (1611.1 ± 400.8 arbitrary units), as compared to each of the other groups: 389.7 ± 14.2 in mice treated with PBS-liposomes and IgG; 758.9 ± 171.4 in mice treated with PBS-liposomes and Jo2; and 444.4 ± 40.5 in mice treated with clodronate liposomes and IgG (Figure 6A). As a separate measurement of apoptosis, we counted the number of cells staining positive by TUNEL in lung tissue sections (Figure 6B). There was a trend towards greater number of TUNEL-positive nuclei in the lungs of the mice treated with clodronate liposomes and Jo2, but this did not reach statistical significance.

We and others have postulated that alveolar epithelial cell apoptosis results in epithelial disruption and increase in the permeability of the epithelial barrier to proteins. The lung permeability response was assessed by measuring the BALF total protein and IgM concentrations. The BALF total protein concentration was 356.5 ± 60.0 µg/ml in the Clod + Jo2 mice, and this was significantly increased with respect to each of the other treatment groups (56.6 ± 2.3 µg/ml, PBS + IgG; 69.3 ± 11.8 µg/ml, PBS + Jo2; 165.3 ± 19.6 µg/ml, Clod + IgG, p < 0.05) (Figure 7A). The BALF total protein concentration was also significantly increased in the Clod + IgG group as compared to the PBS + IgG group (p < 0.05). The BALF concentration of IgM followed a similar pattern and was highest in the

![Figure 7](image_url). Total BALF protein (A) and IgM (B) concentrations in mice treated with intratracheal liposomes containing PBS or clodronate, followed 24 hr later by intratracheal instillations of Jo2 mAb or control IgG mAb, 2.5 µg/g and studied 18 hr later. n=6/group, except for PBS + IgG, n=5.
Contrary to our expectations, there was no apparent increase in the permeability markers in response to Jo2 mAb in the PBS-liposome treated mice.

**Jo2 mAb causes KC release from mouse lung epithelial cells in vitro**

Because the *in vivo* studies suggested that Jo2 mAb induced a pro-inflammatory cytokine response in the macrophage depleted mice, we investigated the cytokine response of mouse lung epithelial cells to Jo2 stimulation. We used MLE-12 cells, a lung epithelial cell line with several type II features which expresses Fas. As a representative cytokine we measured KC, a murine functional homolog of IL-8. The KC concentrations were measured in supernatants from MLE-12 cells after 18 hr incubation with serial concentrations of Jo2 mAb. The release of KC increased proportionally in response to increasing concentrations of Jo2 (Figure 8A). Treatment with the pan-caspase inhibitor ZVAD-fmk did not abrogate KC release, indicating that the induction of KC release was independent of caspase activation. MLE-12 are relatively resistant to Fas-induced apoptosis.

As expected, incubation of the MLE-12 cells with Jo2 mAb caused no effect on cell survival, as determined by Alamar blue assay (Figure 8B). Interestingly, TNF-α, IL-1β, MIP-2 and MCP-1 were all below the limit of detection of the assay.

![Figure 8](image.png)

**Figure 8.** Concentrations of KC in the supernatant of MLE-12 cells incubated with serial concentrations of a control IgG (black), Jo2 mAb (grey) or Jo2 mAb plus the broad caspase inhibitor zVAD (100µM) (dark gray) (A). Cells incubated with VP16 (100µM) serve as positive control. Panel B shows cell survival in the same cells, as determined by the alamar Blue assay. The figures show data from at least 3 independent experiments. Data is shown as means ± SEM; * p<0.05 as compared with cells incubated with control IgG at each time point.
DISCUSSION

The main goal of this study was to determine whether resident alveolar macrophages are required for the development of Fas-induced lung inflammation in mice. Our main findings are that Fas activation induces cytokine release and neutrophilic alveolitis in mice deficient in alveolar macrophages, and that mouse epithelial cells release the chemokine KC in response to Fas activation in vitro. These findings suggest that the pro-inflammatory function of the Fas/FasL system plays an important role in the development of Fas-mediated lung injury, and point towards a prominent role of the epithelium in the cytokine response to Fas activation.

Studies investigating the role of the Fas/FasL system in the development of acute lung injury have focused primarily on its role as a pro-apoptotic system (reviewed in 17). However, in addition to apoptosis, activation of Fas may trigger pro-inflammatory pathways through activation of NF-κB 39. The importance of the pro-inflammatory properties of the Fas/FasL system has been highlighted by a number of independent studies demonstrating that activation of Fas in the lungs of mice by either recombinant sFasL or activating antibodies is followed 3 to 24 hr later by a neutrophilic alveolitis associated with increased concentrations of pro-inflammatory cytokines including TNF-α, IL-1β, KC, MIP-2, GM-CSF, IL-5, and IFN-γ 18-21,23,40. This inflammatory response requires the presence of a functioning Fas receptor in the lungs and is prevented by the administration of pharmacological inhibitors of the Fas/FasL system 19,22,40. To investigate the magnitude of the response, Wortinger et al. directly compared the lung cytokine response to intratracheal instillations of recombinant human FasL (500 ng/mouse) and E. coli LPS (2 µg/mouse) at 3, 6 and 24 hr after instillation, and found that at all times tested, the BALF concentrations of GM-CSF, IL-1β, IL-5, IFN-γ and TNF-α were higher or similar in the FasL-treated mice as compared to the LPS-treated mice 40. Although the amount of FasL used in the Wortinger study was several orders of magnitude higher than the amount of LPS, the Fas/FasL system appears to play a role in lung inflammation induced by a number of noxae, including immune complexes, cecal ligation and puncture, inhaled bacteria and surprisingly, LPS itself 20-22,24.

The observation that Fas activation in the lungs is associated with an inflammatory response led to the question whether Fas signaling can trigger cytokine release by lung cells. Initial studies showed that human and murine macrophages do not become apoptotic in response to Fas ligation, but instead release pro-inflammatory cytokines 8. Specifically, human monocyte-derived macrophages release TNF-α and IL-8 in response to the Fas-activating mAb CH11, and murine alveolar macrophages release KC and MIP-2 in response to human recombinant sFasL 8,40. These findings led us and others to propose the hypothesis that the Fas/FasL system contributes to lung inflammation in vivo by inducing cytokine release by alveolar macrophages. However, this hypothesis has been challenged by two separate studies. First, in a study designed to test whether Fas induces cytokine release by macrophages in vivo, we created chimeric mice expressing Fas in either their myeloid cells or their non-myeloid cells 26. Contrary to the hypothesis, we
found that only those mice expressing Fas in non-myeloid cells developed inflammation in response to the Jo2 antibody, and this was true for both the neutrophilic response and the cytokine response. Second, in a later study, Perl et al. showed that intratracheal instillation of Jo2 mAb induces release of KC, MIP-2 and MCP-1 in mice carrying the CSF1op mutation, even though these mice are deficient in monocytes and macrophages because they lack expression of colony stimulating factor-1. However, these studies are not definitive, because the chimeric mice had been subject to whole body irradiation, which may have modify the populations of immune cells in the lungs; and the CSF1op mice have macrophages derived from non-monocytic populations, and show normal responses to inflammatory stimuli thought to depend on macrophages, such as LPS.

To further investigate the role of alveolar macrophages in Fas-induced pulmonary inflammation, we used a model of macrophage depletion induced by instilling clodronate-containing liposomes into the lungs of mice. Clodronate liposomes cause macrophage depletion by a mechanism involving competitive inhibition of ADP/ATP translocase and subsequent apoptosis. In our study, treatment with Jo2 mAb was followed by neutrophilic inflammation and increased concentrations of several pro-inflammatory cytokines, despite the reduction in alveolar macrophages by clodronate-liposome instillations. Surprisingly, the neutrophil and cytokine responses to Jo2 mAb were actually enhanced by macrophage depletion. These findings suggest that the activation of pro-inflammatory pathways induced by Jo2 was not primarily dependent on alveolar macrophages.

The observation that lung cytokine release and neutrophilic inflammation in lung injury in vivo can occur when alveolar macrophages are depleted is important because it suggests that other cell type(s) in the lung can promote inflammation. In particular, the alveolar epithelium may be an important source of pro-inflammatory cytokines during lung injury. Studies performed in vitro show that primary human alveolar type II cells stimulated with LPS release CXC and CC chemokines, including MCP-1, GRO and IL-8. Additional experiments performed on rat primary type II cells and the human neoplastic type II cell line A549 confirm that alveolar epithelial cells can release IL-6 and IL-8, in response to IL-1β, TNF-α, and conditioned supernatants from LPS-treated macrophages. In the present study, mouse lung epithelial cells released the neutrophilic chemokine KC in response to Jo2 mAb in vitro. This further supports the hypothesis that the lung neutrophilic response in Fas-mediated lung injury may depend at least partly on cytokine release by alveolar epithelial cells.

An unexpected finding of the present study is that macrophage depletion seemed to worsen Fas-induced lung injury. Other investigators have found a similar enhanced lung inflammation and injury by macrophage depletion in different models of experimental lung injury. Using a rat model of aerosolized LPS, Elder et al. found that clodronate-containing liposome treatment led to a 5-fold increase in BALF PMN counts, as compared to saline liposomes. These findings have been reproduced by Beck-Schimmer et al. using a similar
rat model of LPS-induced lung injury and by Nakamura et al. in a rat model of ischemia-reperfusion \cite{48,49}. In addition, a delayed but more extensive lung neutrophilic response associated with increased mortality occurs in macrophage-depleted mice infected with *Pseudomonas aeruginosa* \cite{50}. Thus, several studies using models of lung injury that vary from activation of one single pathway (e.g. Fas in the present study) to instillation of LPS or live bacteria, have found that macrophage depletion can be associated enhanced lung injury. Knapp et al. have suggested that deficient phagocytosis and degradation of apoptotic PMNs due to macrophage depletion may be one mechanism of such enhanced lung injury \cite{51}. However, other studies have found that macrophage depletion with clodronate liposomes results in attenuation of lung injury following LPS administration, ischemia-reperfusion, experimental sepsis and mechanical ventilation \cite{52-58}. Thus, a possible explanation is that the relative contribution of the epithelium and macrophages to the production of pro-inflammatory cytokines is dependent on the initial injury to the lung, with the macrophages acting as immunomodulatory cells. Additional studies are needed to clarify the mechanisms linking macrophages and the inflammatory response in lung injury as well as the specific contribution of the Fas/FasL system to epithelial cytokine release.

Our study has several limitations. First, the administration of clodronate liposomes did not result in a complete depletion of macrophages in the BALF. It could be argued that the residual macrophage population could be sufficient to induce a cytokine response. However, studies in which a comparable reduction in the of alveolar macrophages was achieved with clodronate liposomes treatment have shown impaired cytokine responses \cite{57}. As mentioned above, this suggests that the role of resident macrophages on cytokine production may depend on the cause of the lung injury, and further studies are needed to determine this issue. Another concern is that recruitment of highly pro-inflammatory monocytes could have explained the inflammatory responses to Jo2 mAb in the macrophage-depleted mice. However, we did not find evidence for an increase in the proportion of F4/80+, CCR2+ cells, which have been associated with increased pro-inflammatory activity \cite{33,37,59}. Finally, it is possible that the clodronate liposomes “primed” the lungs for additional injury. Our data shows that clodronate liposomes induced a mild neutrophilic response at baseline, which could have been magnified by the subsequent administration of Jo2. However, even if this was the case, our main conclusion that resident alveolar macrophages were not the primary source of pro-inflammatory cytokines remains valid.

In summary, depletion of alveolar macrophages by clodronate liposomes does not prevent, and may enhance, the lung cytokine and neutrophilic responses of mouse lungs to Fas activation *in vivo*. In addition, Fas activation with Jo2 mAb induces release of the chemokine KC by mouse lung epithelial cells *in vitro*. We conclude that the lung inflammatory response to Fas activation is not primarily dependent on alveolar macrophages and may instead depend on cytokine release by alveolar epithelial cells. These data are consistent with the interpretation that the alveolar epithelium may be an important source of pro-inflammatory cytokines during early acute lung injury.
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Potential Role of Soluble TRAIL in Epithelial Injury in Children with Severe RSV Infection

Reinout A. Bem¹
Albert P. Bos¹
Roelie M. Wösten-van Asperen ¹
Martijn Bruijn¹
Rene Lutter²
Martin R. Sprick³
Job B.M. van Woensel¹

¹ Pediatric Intensive Care Unit, Emma Children’s Hospital; ² Departments of Pulmonology and Experimental Immunology, and ³ Laboratory of Experimental Oncology and Radiobiology Academic Medical Center, Amsterdam, The Netherlands

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ABSTRACT

Lower respiratory tract infection by respiratory syncytial virus (RSV) is a frequent cause of acute lung injury in young children and infants. Studies in adults and animals suggest that tumor necrosis factor receptor (TNFR) ligands may mediate lung injury by causing apoptosis of epithelial cells. The main goal of the present study was to determine whether the TNF-related apoptosis-inducing ligand (Apo2L/TRAIL) pathway may be implicated in epithelial injury during severe RSV infection in children. We report elevated levels of soluble (s)TRAIL released by leukocytes in bronchoalveolar lavage fluid (BALF) of patients with RSV-associated respiratory failure (n=22) as compared to mechanically ventilated patients without pulmonary illness (n=7). Primary bronchial epithelial cells of children without pulmonary disease obtained by non-bronchoscopic cytobrushing expressed both death receptors TRAIL-R1 and -R2, and were found to be susceptible for cell death by human recombinant sTRAIL in vitro. Furthermore, BALF from a RSV patient induced cell death in these cells, which was partly attenuated by inhibiting TRAIL signaling. These data suggest that the TRAIL pro-apoptotic pathway may contribute to lung epithelial injury in severe RSV infection in children.
INTRODUCTION

Lower respiratory tract infection (LRTI) by respiratory syncytial virus (RSV) remains a frequent cause of acute respiratory failure in young children and infants worldwide. RSV-LRTI may present as bronchiolitis with obstructive airway symptoms, and/or pneumonia, involving the alveolar compartment. Furthermore, severe RSV-LRTI accounts for up to 20% of the admissions for acute lung injury (ALI) or the acute respiratory distress syndrome (ARDS) in the pediatric intensive care unit. The precise mechanisms by which RSV infection progresses to acute respiratory failure in previously healthy children are largely unknown. Currently, treatment of severe RSV-LRTI is limited to supportive measures.

Histopathology studies in children have shown that fatal RSV disease is associated with marked expression of pro-apoptotic markers, such as active caspase-3, in airway and alveolar epithelial cells. However, it is difficult to interpret these findings: on the one hand it may indicate RSV disease can progress despite an ongoing host defense mechanism of apoptosis of infected cells, on the other hand it may suggest extensive and imbalanced cell death during RSV infection leads to enhanced lung injury. In fact, increased caspase-3 activity in lung epithelial cells is also found in adult patients who died of ALI/ARDS, and numerous animal models of ALI/ARDS, including a murine model for severe RSV infection, have shown that lung injury is associated with enhanced lung epithelial cell apoptosis. More importantly, several of these animal studies also demonstrated that strategies inhibiting pro-apoptotic signalling may attenuate lung inflammation, alveolar-capillary permeability and histopathological alterations.

These observations suggest that under certain circumstances activation of pro-apoptotic pathways in the lungs can contribute to lung injury, and this may also play a role in the pathogenesis of severe RSV-LRTI.

An important pro-apoptotic pathway is triggered by the activation of specific membrane “death” receptors belonging to the tumor necrosis factor receptor (TNFR) superfamily. One of the ligands for this TNFR pathway is TNF-related apoptosis-inducing ligand (Apo2L/TRAIL), which initiates caspase activation upon ligation of either one of two TNFR members: TRAIL-R1 and -R2. TRAIL is a type 2 transmembrane protein, but a soluble form (sTRAIL) can be generated after cleavage of the extracellular domain by cysteine proteases or through secretion in microvesicles. In the lungs, TRAIL is potentially expressed by a number of different activated leukocytes, including monocytes/macrophages, neutrophils and lymphocytes. In addition, expression of TRAIL and its receptors has been found in human alveolar septa and bronchial epithelium. Interestingly, RSV infection of human carcinomic alveolar basal epithelial cells (A549 cell line) in vitro upregulates the surface expression of TRAIL-R1 and -R2, which suggests the TRAIL pathway may contribute to host defense by limiting RSV replication and spread. However, in a recent study by Herold et al. mice with influenza virus pneumonia and TRAIL deficient macrophages or treated with anti-TRAIL mAb showed decreased lung...
epithelial cell apoptosis, together with decreased lung permeability and mortality, despite delayed viral clearance. These results suggest that TRAIL-induced lung epithelial cell death may be an important pathogenic mechanism of lung injury in severe viral lung infection.

In the present study we investigated the hypothesis that the activation of the TRAIL pathway is a potential mechanism of lung epithelial injury in severe RSV-LRTI in children. We measured levels of sTRAIL in bronchoalveolar lavage fluid (BALF) of children receiving mechanical ventilation for RSV-LRTI, and explored the susceptibility of normal primary bronchial epithelial cells of children to TRAIL-induced cell death in vitro.

72 METHODS

All patient sampling protocols were approved by the Academic Medical Center ethical committee and informed consent was obtained from parents.

Bronchoalveolar lavage fluid samples

Patients. Between November 2007 and January 2009 BALF samples were obtained from 22 children receiving mechanical ventilation for RSV-LRTI and 7 age-matched patients receiving mechanical ventilation for non-pulmonary conditions. Infection with RSV was proven by direct immunofluorescence assay (Imagen, DakoCytomation, UK) of nasopharyngeal aspirate. RSV patients with bilateral infiltrates on chest radiography and a PaO$_2$/FiO$_2$ ratio of < 200 mmHg in the absence of cardiac failure were designated to fulfil the American-European consensus criteria of ARDS.

Sampling and processing. BALF was obtained on the day of start of mechanical ventilation, and in the RSV patients also on day 2 and 4 thereafter. BALF was obtained by three subsequent instillations of 1 ml/kg of 0.9% saline through a wedged suction catheter passed through the endotracheal tube. After each instillation fluid was suctioned (mean % recovery ± SE: 36.9 ± 1.8 for RSV patients and 28.7 ± 3.5 for controls). The last 2 samples were pooled and 10 min centrifuged at 450 x g. Supernatant was aspirated, aliquoted and stored at -80°C. The mucoid cell pellet was resuspended in 10 mM dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, MO) in 25 mM Hepes (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) pH 8.0 at 4°C followed by 10 minutes mixing. After again 10 min centrifugation at 450 x g the cell pellet was resuspended in PBS and total white blood cells (WBC) were counted in a Bürker bright line counting chamber. Air dried cytospins were stained with Romanovsky (Diff-Quick) and differential WBC counts were obtained by counting 200 leukocytes using a standard light microscope.

In several patients the remainder of the BAL cells was incubated with RPMI containing L-glutamine (Invitrogen Ltd, Paisley, UK), supplemented with 0.1% penicillin/streptomycin...
and 10% fetal bovine serum (Invitrogen Ltd), for 18 hr at 37°C, 5% CO₂, after which the supernatant was collected.

**ELISA.** sTRAIL in BALF and cell supernatants was measured by ELISA (R&D systems, Minneapolis, MN), according to the manufacturer's description.

**FACS analysis.** For detection of TRAIL expression on BAL cells in 10 RSV patients, BAL cells in FACS buffer (0.5% BSA, 0.02% potassium-EDTA in PBS) containing 10% normal human serum were labeled with APC-labeled anti-CD3 or anti-CD14 (BD Biosciences, Franklin Lakes, NJ), FITC-labeled anti-CD16 (Sanquin, the Netherlands) and -56 (BD Pharmingen) and PE-labeled anti-TRAIL mAb (BD Biosciences) or a PE-labeled isotype control (BD Biosciences). The gating strategy used has been described previously. The FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (Tree Star Inc, Ashland, OR) were used for analysis.

**Primary lung epithelial cell cultures**

**Patients.** Primary bronchial epithelial cells were obtained by non-bronchoscopic cytobrush sampling in 6 children (age 0-4 yr) who were endotracheally intubated for elective minor surgery for non-pulmonary disorders.

**Sampling and culturing.** Briefly, a sheathed cytology brush (2.4 mm, Olympus, Hamburg) was introduced through the endotracheal tube directly after intubation. In wedge position the sheath and brush were withdrawn ~2 cm after which gentle brush sampling was performed over a range of ~2 cm. The obtained cells were shaken of the brush and cultured at 37°C, 5% CO₂ in collagen- (Vitrogen-100, Cohesion Technologies, Palo Alto, CA) coated cell culture plates (Corning Costar, Schiphol-Rijk, The Netherlands) in BEGM-Bulletkit bronchial epithelial medium containing the following supplements/growth factors: bovine pituitary extract, hydrocortisone, hEGF, epinephrine, transferrin, insulin, retinoic acid, triiodothyronine, and gentamicin/amphotericin-B (Cambrex Cooperation, NJ) and ciproxin (Bayer BV, Mijdrecht, The Netherlands). After ~2 wks of culturing and before passage 2-3, the cells were used for experiments at ~70-80% confluency.

**Virus exposure.** RSV-A aliquots were a kind gift from L. van der Hoek, Department of Experimental Virology AMC, Amsterdam, The Netherlands. Cells at ~70-80% confluency in 24-well plates (Corning Costar) were exposed to a total of 3x10⁷ copies RSV-A, as determined by qPCR. This dose was based on previous experiments which showed intracellular RSV replication by direct immunofluorescence assay (Imagen, DakoCytomation, UK) at day 6-7 post-infection.

**Immunocytochemistry.** Cytospin preparations were fixed in 4% paraformaldehyde, treated with peroxidase block (Envision system, DAKO, Carpinteria, CA) and blocked with 3% bovine
serum albumin in PBS/TritonX-100 0.1%. Thereafter, the cells were exposed to one of the following primary antibodies: mouse anti-human cytokeratin 1-8, 10, 13-16 and 19 mAb (AE1/AE3 clone, DAKO), anti-human TRAIL-R1/DR4 or anti-human TRAIL-R2/DR5 (Axxora, San Diego, CA) for 1 hour at RT, followed by incubation with labelled polymer-HRP anti-mouse and AEC+ substrate chromogen (Envision system, DAKO) according to the manufacture’s description. The cells were counterstained with haematoxylin.

FACS analysis. For detection of TRAIL-R1 and -R2 expression cells in FACS buffer (0.5% BSA, 0.02% potassium-EDTA in PBS) were labeled with anti-human TRAIL-R1 or anti-human TRAIL-R2 (Axxora, San Diego, CA), and detected with RPE-labelled rabbit anti-mouse Ab (DAKO). The FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (Tree Star Inc, Ashland, OR) were used for analysis.

Cytotoxicity assays. To investigate the susceptibility to TRAIL induced apoptosis in vitro, cells were exposed to 2 different forms of human recombinant sTRAIL: superkillerTRAIL (Axxora, San Diego, CA) diluted in 20mM HEPES, pH 7.4, containing 300mM NaCl, 0.01% Tween 20, 1% sucrose and 1mM DTT or FLAG-TRAIL (Axxora) either with or without M2-anti-FLAG antibody at a ratio of 2 µg antibody to 1µg TRAIL to establish crosslinking. To determine the cytotoxicity of sTRAIL in BALF, BALF was concentrated 40-fold by ultrafiltration (Centricron Ultracel YM-10, Millipore, Billerica, MA), sterile-filtered, and then added to the cells at a 1:50 dilution. For specific blocking of TRAIL-mediated effects, the BALF-concentrate was incubated with 10 ug DR5-Fc fusion protein (R&D systems, Minneapolis, MN) for 15 min at room temperature before addition to the cells. Cell death was determined with a flow-cytometer upon incubation of the cells with FITC-labeled Annexin V (Nexins, Kattendijke, The Netherlands) or propidium iodide.

Statistical analysis
Statistical analysis was performed with SPSS 16.0 software. Not normally distributed data are expressed as medians and were analyzed by using the Mann-Whitney U test for differences between groups. For normally distributed data, a student’s t-test or paired t-test was used to

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<table>
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<td>16 (73)</td>
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<td>22.5 (28.9)</td>
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<td>72 (12-89)</td>
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compare means. Proportions in the patient groups were compared by Fisher’s exact test. A two-sided p value of < 0.05 was considered statistically significant.

RESULTS

Patient characteristics
The baseline characteristics of the patients are shown in Table 1. Reasons for admission and mechanical ventilation of patients without pulmonary/RSV disease were postoperative abdominal surgery (n=5), meconium ileus associated with respiratory distress (n=1) and laryngeal granuloma (n=1). The (first) BALF sample was obtained at a mean (±SD) of 18.3 (±15.0) hr after start of mechanical ventilation in the patients without pulmonary/RSV disease,

![Figure 1](131x335) 10
![Figure 1](127x367) 100
![Figure 1](123x399) 1000
![Figure 1](118x431) 10000

no lung disease RSV
day start of MV peak

![Figure 1](113x217) sTRAIL (pg/ml) in BALF

![Figure 1](113x217) start of MV 2 4
day

![Figure 1](113x217) sTRAIL (pg/ml) in BALF

![Figure 1](113x217) no ARDS ARDS

Figure 1. A, levels of sTRAIL (pg/ml) in BALF from children without pulmonary/RSV disease (n=7) as compared to children with RSV infection (n=22) on the day of start of mechanical ventilation (MV) and individual peak levels during the course of RSV infection. * p <0.05 (Mann-Whitney U test) as compared to children without pulmonary/RSV disease. B, levels of sTRAIL (pg/ml) in BALF on the day of start of mechanical ventilation from children with or without ARDS during the course of RSV infection (p=0.07 by t-test). C, levels of sTRAIL (pg/ml) in BALF from children during the course of RSV infection. Data are shown as box plots depicting the median, interquartile range and range.
and at 15.2 (±5.9) hr in the RSV patients (p = 0.4). The mean (±SE) tidal volume at time of BALF sample was 8.0 (±0.5) for patients without pulmonary/RSV disease, as compared to 6.6 (±0.3) for RSV patients. In total, 5 of the RSV patients had a history of preterm birth, and 3 of the RSV patients had a pulmonary or cardiac abnormality. Seven (31%) of the RSV patients fulfilled the criteria of ARDS during admission.

Elevated levels of sTRAIL in the lungs of children with severe RSV infection
On the day of start of mechanical ventilation, the median (IQR) level of sTRAIL in BALF of the RSV patients was 298 (287) pg/ml, as compared to 147 (110) pg/ml in the patients without pulmonary disease (p< 0.05) (Figure 1A). RSV patients who fulfilled the criteria of ARDS during the course of RSV disease had a median (IQR) sTRAIL level of 332 (503) pg/ml in BALF, as compared to 253 (229) pg/ml in RSV patients without ARDS, but this difference did not reach statistical significance (Figure 1B). Because it was unknown at what time point actual infection by RSV had occurred in the patients, we also examined levels of sTRAIL in BALF at day 2 and 4 after start of mechanical ventilation (Figure 1C). Although levels of sTRAIL within patients at the different time points were variable, the median levels of sTRAIL remained high during the course of RSV infection. The median (IQR) of the individual peak levels of sTRAIL in BALF of the RSV patients was 393 (299) pg/ml (Figure 1A). There was no correlation between levels of sTRAIL in the BALF and tidal volume or duration of mechanical ventilation (data not shown).

sTRAIL is released from lung leukocytes
In viral infected lungs, TRAIL may be expressed by several activated leukocytes, including monocytes/macrophages, neutrophils and lymphocytes. Specifically, activated monocytes and neutrophils have been shown to release sTRAIL. To determine whether sTRAIL is released by lung leukocytes in RSV infection, BAL cells were isolated by centrifugation from the BALF from RSV patients and incubated in RPMI medium containing 10% fetal bovine serum. The supernatants of the BAL cells collected after 18 hr incubation all contained sTRAIL (mean ± SEM level per 5x10^6 cells: 128 ± 28 pg/ml for RSV patients, and 32 ± 24 pg/ml for patients without pulmonary disease) (Figure 2A). RPMI medium and fetal bovine serum did not contain human sTRAIL.

In the lungs of mice infected with influenza virus marked expression of TRAIL is found predominantly in macrophages. In line with this observation, we found evidence for expression of membrane-bound TRAIL in CD14^+ BAL cells (monocytes/macrophages) during RSV infection by FACS analysis (Figure 2B). In contrast, no clear expression of TRAIL was found in CD3^+ BAL cells (T-lymphocyte population) and CD3^-CD16^-56^ BAL cells (NK cell population) (Figure 2B).
Primary normal bronchial epithelial cells of children express TRAIL-R1 and -R2, which is increased by RSV infection

Activation of TNF death receptor-ligand systems, including the TRAIL pathway, has been implicated in the development of lung epithelial injury in vivo. This led us to hypothesize that TRAIL in the lungs of children with severe RSV infection may activate the death receptors TRAIL-R1 and/or -R2 on epithelial cells. Because epithelial cells in the developing lungs of young children may differ from primary adult or cancer-derived cell lines we isolated and cultured bronchial epithelial cells of children, age 0-4 year, by non-bronchoscopic cytobrushing. More than 98% of the cells obtained after culturing for a maximum of ~2 weeks stained positive for epithelial specific cytokeratins (data not shown). Moreover, primary bronchial epithelial cells expressed both TRAIL-R1 and -R2 death receptors as determined by immuncytochemistry and FACS analysis (Figure 3A and 3B).

In vitro exposure to RSV and influenza virus has been reported to increase the expression of TRAIL receptors in A549 and mouse alveolar epithelial cells respectively. Similarly, exposure of primary bronchial epithelial cells of children to RSV-A in vitro resulted in increased expression of both TRAIL-R1 and -R2 at 6 days post-infection (Figure 3C). Intracellular RSV replication in primary bronchial epithelial cells at this time point was confirmed by direct immunofluorescence assay. The pattern of expression of TRAIL-R1
and TRAIL-R2 8 days post-infection differed between samples, as we observed either a moderate further increase or down regulation (data not shown).

**Primary bronchial epithelial cells of children are susceptible to TRAIL-induced cell death *in vitro***

The susceptibility to TRAIL-induced cell death of primary bronchial epithelial cells of children *in vitro* was analyzed in three different ways. First, we exposed cells to human recombinant superkillerTRAIL (Axxora), which is a form of sTRAIL that has been cross-linked to enhance its cytotoxic potential. As compared to exposure to dilution buffer only, primary bronchial epithelial cells exposed to superkillerTRAIL at a concentration of 50 ng/ml for 6 hr showed

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**Figure 3.** A, immunocytochemistry for TRAIL-R1 and -R2 of cytopsins of primary bronchial epithelial cells of children (magnification 500x). B, representative examples of FACS histogram plots of primary bronchial epithelial cells of children stained for membrane bound TRAIL-R1 and -R2. Negative control (grey surface plot) is without primary antibody. C, representative examples of FACS histogram plots for membrane bound TRAIL-R1 and -R2 staining on primary bronchial epithelial cells of children, 3 and 6 days post-infection (dpi) with RSV-A in vitro. Uninfected cells are shown in grey surface plots.
Figure 4. A, fold increase in the percentages of annexin V positive cells upon 6 hr exposure to superkillerTRAIL, as compared to cells treated with dilution buffer only. Experiments were performed in duplicates with primary bronchial epithelial cells obtained from three different children. B, example of FACS histogram plots from primary bronchial epithelial cells stained for annexin V upon 6 hr exposure to superkillerTRAIL, as compared to cells treated with dilution buffer only. C, percentages of PI positive primary bronchial epithelial cells from one child upon 48 hr exposure to non cross-linked FLAG-TRAIL with or without M2-anti-FLAG antibody to establish crosslinking. D, percentages of PI positive cells upon 48 hr exposure to non-cross linked FLAG-TRAIL (33 ng/ml) or concentrated BALF from a RSV patient with or without DR5-Fc to neutralize TRAIL signalling. Experiments were performed in duplicates with primary bronchial epithelial cells obtained from two different children. * p <0.05 (paired t-test). Data are shown as bars depicting the mean and standard error. E, representative light microscopy photograph showing the apoptotic morphological appearance of primary bronchial epithelial cells of children in TRAIL cytotoxicity assays (zoomed in 250x magnification). Inset shows normal unexposed control cells (250x magnification).
a 2-fold increase in the percentage of annexin V-positive cells (Figure 4A and 4B). Next, we exposed primary bronchial epithelial cells to non cross-linked human recombinant FLAG-sTRAIL without or with M2-anti-FLAG antibody to establish crosslinking. Non cross-linked sTRAIL at a concentration of 33 ng/ml and higher also clearly induced cell death in primary bronchial epithelial cells, although the susceptibility to this form of sTRAIL appeared much lower than to the cross-linked sTRAIL (Figure 4C). Finally, we exposed primary bronchial epithelial cells to a BALF sample obtained from a RSV patient (RSV-BALF). This BALF sample contained sTRAIL at a concentration of approximately 600 pg/ml as determined by ELISA. The RSV-BALF sample was concentrated 40-fold and used at a dilution of 1:50 to get a biologically relevant concentration of sTRAIL, while culturing in a normal volume of medium, and thus strongly reducing background cell death. Exposure to RSV-BALF for 48 hr induced a 5-fold increase in cell death in primary bronchial epithelial cells which was partly (~30%) attenuated by the addition of DR5-Fc which binds and neutralizes TRAIL (Figure 4D). Cell death in the above mentioned experiments was accompanied by the appearance of apoptotic morphological features such as cell shrinkage, rounding, cell membrane blebbing and detachment (Figure 4E).

Neutralization of TRAIL induced cell death by DR5-Fc was found to be complete in the highly TRAIL-sensitive BJAB cell line (Supplemental figure 1A), and neither DR5-Fc or human IgG exerted direct cytotoxicity in BJAB cells or the primary bronchial epithelial cells (Supplemental figure 1A-B).

DISCUSSION

The goal of this study was to determine whether severe RSV infection in children is associated with local activation of the pro-apoptotic TRAIL pathway, and whether this may be a mechanism of lung epithelial injury. We found elevated levels of sTRAIL in BALF of children receiving mechanical ventilation for RSV-LRTI. Normal primary bronchial epithelial cells of children expressed both death receptors TRAIL-R1 and -R2, and human recombinant sTRAIL as well as sTRAIL in RSV-BALF induced cell death in these cells in vitro. These findings suggest that sTRAIL may contribute to lung epithelial injury in children with severe RSV infection.

The present study extends our insight into cell death mechanisms in lung injury. Pro-apoptotic signaling is an essential process in growth, development and homeostasis in the lungs. However, accumulating evidence links enhanced activation of specific TNFR death receptors on lung epithelial cells to the pathophysiology of ALI/ARDS. For example, Albertine et al. found that alveolar wall cells of patients with fatal ALI/ARDS show prominent immunohistochemical staining for apoptotic markers such as terminal dUTP nick-end labeling, caspase-3, Bax, and p53, as well as the TNFR Fas. Furthermore, increased levels of soluble Fas ligand (sFasL) are present in BALF of patients with fatal
ALI/ARDS, and this has been implicated in apoptosis of primary lung epithelial cells of adults in vitro. Numerous studies with animal models for ALI/ARDS using (in)direct lung hits such as LPS, live bacteria, cecal ligation and puncture, hemorrhagic shock, mechanical ventilation and hyperoxia have shown extensive apoptosis of lung epithelial cells is associated with dysfunction of the alveolar-capillary barrier and histopathological injury. Interestingly, the lung injury in several of these animal studies can be attenuated by pharmacological apoptosis inhibitors or gene targeting of the Fas/FasL system, highlighting the relevance in the search for new therapeutic strategies.

Activation of TNFR-mediated apoptosis causing epithelial injury appears to be a general lung injury response, although the responsible mechanisms remain incompletely understood and may actually differ between injurious events such as mechanical ventilation and bacterial- or viral infection. We hypothesized that activation of the TNFR system may also play a role in the development of severe RSV infection in children, as characteristic lung pathological changes of fatal RSV-LRTI include small airway entrapment of apoptotic cellular debris and increased expression of active caspase-3 and Fas in airway and alveolar epithelial cells. However, previously, we have failed to detect sFasL in BALF of children with severe RSV infection by 2 different commercially available ELISAs (unpublished data), although there are concerns that this may in part be related to low specific antibody affinity as compared to anti-FasL antibodies used in other previous studies. In the present study we investigated the alternative, highly homologous TNFR-ligand system involving TRAIL and its death receptors -R1 and -R2. We found increased levels of sTRAIL, in the range of 0.1-1.5 ng/ml, in the lungs of children with severe RSV-LRTI. sTRAIL was released by lung leukocytes, and caused apoptosis of primary epithelial cells of children at a biologically relevant concentration in vitro. Interestingly, a recent study by Herold et al. showed a critical role for TRAIL-induced apoptosis of lung epithelial cells in disease pathogenesis of influenza virus pneumonia in mice. They showed that treatment with specific anti-TRAIL mAb antibodies which inhibited TRAIL signaling causes decreased lung epithelial cell death, lung permeability and mortality. Taken together, these findings suggest that the activation of the TRAIL pathway may be a mechanism of epithelial injury in severe viral infection, including influenza virus and RSV.

Several activated leukocytes, including macrophages, neutrophils and lymphocytes, are a potential source of TRAIL expression in the lungs. Monocytes/macrophages and neutrophils activated by type I interferon or LPS release sTRAIL with intact pro-apoptotic activity by signaling through both TRAIL-R1 and -R2. sTRAIL is released through secretion by microvesicles, but can also be generated by cleavage of the extracellular domain of membrane bound TRAIL by cysteine proteases. In the aforementioned study by Herold et al. TRAIL expression in the lungs during influenza virus infection in mice was confined to macrophages. In line with this observation we found evidence for expression of membrane bound TRAIL on lung monocytes/macrophages, and no clear expression in CD3+ and NK lymphocytes. However, because we investigated TRAIL...
expression in a limited number of RSV patients and did not include a specific neutrophil marker we cannot draw firm conclusions from this.

To model epithelial cells in the lungs of children we cultured primary bronchial epithelial cells from children without pulmonary disease by non-bronchoscopic cytobrushing. An advantage of this in vitro model is that the apoptotic responses of these cells may reflect the developing pediatric lungs to a greater extent than primary adult or cancer-derived lung epithelial cells. Although in severe RSV infection both airway and alveolar epithelial cells appear to undergo extensive apoptosis, our in vitro model may not necessarily be representative for the apoptotic responses of alveolar epithelial cells. Even more pronounced apoptotic responses of distal as compared to proximal lung epithelial cells upon activation of Fas have been reported, but at this moment we can only speculate that this may be true for TRAIL as well.

In this study, primary lung epithelial cells of children were exposed to three different forms of sTRAIL: non-cross linked and cross-linked human recombinant sTRAIL, and finally sTRAIL in RSV-BALF at a biologically relevant concentration. In a study by Wajant et al. cross-linked sTRAIL was found to behave similar to membrane bound TRAIL acting through both TRAIL-R1 and -R2, while non cross-linked sTRAIL like natural sTRAIL activated only TRAIL-R1. However, in a more recent study Tecchio et al. showed that TRAIL released by neutrophils and monocytes can activate both TRAIL-R1 and -R2. Here we show that normal primary lung epithelial cells of children expressed both TRAIL-R1 and -R2, and were susceptible to cell death induced by both non cross-linked and cross-linked recombinant sTRAIL. More importantly, RSV-BALF caused considerable cell death in primary epithelial cells of children, and this was partly attenuated by the addition of DR5-Fc which blocks TRAIL signalling. However, this observation necessitates several considerations. First, apart from sTRAIL a number of different cytokines and enzymes in RSV-BALF (e.g. TNFα, sFasL), and MMPs and other (serine) proteases) may have (in)directly activated cell death programs, and moreover, they may have enhanced the sensitivity of primary epithelial cells of children to TRAIL in vitro. In addition, it is possible that by concentrating the BALF though filtration low molecular weight bioactive material, such as degraded cytokine fragments, with potential effects on cell survival has been lost. Second, actual effective local levels of sTRAIL in the lungs in vivo may be higher, because epithelial lining fluid is diluted in BALF and sTRAIL bound at the surface of target cells may not be washed out. Furthermore, we have to take into account that there are a number of additional factors, including the treatment with mechanical ventilation, that may affect the lung microenvironment including TRAIL/apoptotic susceptibility.

It is important to consider that viral infection itself may sensitize cells to TRAIL-induced cell death by increasing TRAIL receptor expression, as has previously been shown for RSV infected A549 cells by Kotelkin et al. and for influenza virus infected mouse lung epithelial cells by Herold and co-workers. In line with these results we found evidence that in vitro RSV infection increased TRAIL-R1 and -R2 surface expression on normal primary
bronchial epithelial cells of children. This may suggest a complex interplay between host defence against virus infected cells on the one hand, and immunopathogenic mechanisms by sTRAIL against bystander (uninfected) epithelial cells on the other hand. However, in the case of widespread viral infection this theoretical concept of bystander versus viral infected cell, in terms of death of bystander cells is ‘bad’, whereas death of infected cells is ‘good’, may just be too simple. The seemingly beneficial effect of activation of pro-apoptotic pathways specifically directed against viral infected cells may be completely lost when a high number of cells is infected, leading to widespread cell death and therefore serious injury to the lungs and airways. This is supported by the study by Herold et al. which showed that inhibition of the TRAIL pathway attenuates lung injury despite decreased influenza viral clearance in mice. Our results show that sTRAIL may cause death of human airway epithelial cells, either infected by RSV or not, but whether this mechanism contributes to severe RSV disease in vivo remains to be elucidated.

In the present study we did not observe a statistically significant relation between disease severity (ARDS and duration of mechanical ventilation) and the levels of sTRAIL in the lungs. Remarkably, in general the development of ARDS during RSV infection leads to a minor increase in mortality in young children. This suggests that patients admitted for RSV-acute respiratory failure may represent the end of spectrum of RSV disease, regardless of fulfilling the clinical criteria of ARDS, and this withholds us from drawing firm conclusions about our findings in this cohort. The fact that several animal studies have convincingly shown that TNFR ligands, including TRAIL, can under certain circumstances contribute to the development of lung injury, to our believe warrants further research on this topic.

In summary, severe RSV-LRTI in children is associated with elevated levels of sTRAIL in the lungs. Primary lung epithelial cells of children are susceptible for sTRAIL-induced apoptosis in vitro. We speculate that activation of the TRAIL pro-apoptotic pathway is a mechanism of lung epithelial injury in severe RSV infection in children.
REFERENCES


Supplemental figure 1. A, percentages of PI positive BJAB cells upon 48 hr exposure to DR5-Fc (10µg/ml), human IgG (10µg/ml) or human cross-linked recombinant TRAIL (10-1000 µg/ml) with and without DR5-Fc or human IgG. Note the complete neutralization of TRAIL signaling by DR5-Fc. B, percentages of PI positive primary bronchial epithelial cells of children upon 48 hr exposure to DR5-Fc (10µg/ml) or human IgG (10µg/ml). Note the absence of direct Fc- or antibody cytotoxicity.
Activation of the Granzyme Pathway in Children with Severe RSV Infection

Reinout A. Bem
Albert P. Bos
Michael Bots
Angela M. Wolbink
S. Marieke van Ham
Jan Paul Medema
Rene Lutter
Job B.M. van Woensel

1 Pediatric Intensive Care Unit, Emma Children’s Hospital AMC; 2 Laboratory for Experimental Oncology and Radiobiology, Academic Medical Center; 3 Department of Immunopathology, Sanquin Research at CLB and Landsteiner Laboratory, Academic Medical Center; 4 Departments of Pulmonology and Experimental Immunology, Academic Medical Center, Amsterdam, The Netherlands

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ABSTRACT

Granzymes, serine proteases present in granules of effector lymphocytes, are involved in several host immune responses, including the activation of cell death and inflammatory pathways. The main goal of this study was to determine whether the local cell-mediated granzyme pathway is activated during severe respiratory syncytial virus (RSV) lower respiratory tract illness (LRTI) in children. Tracheal aspirates from 23 children with RSV-LRTI and 12 controls without pulmonary disease were analyzed for granzyme (Gr) A and B. Bronchoalveolar lavage fluid samples from 7 children with RSV-LRTI were analyzed for cellular expression of GrB. Levels of GrA and GrB in tracheal aspirate were significantly increased in RSV patients as compared to controls and both granzymes showed preserved activity. Granzyme levels correlated with the total leukocyte counts and IL-8 levels in the airways at several time points. However, no correlation between granzyme levels and release of caspase-cleaved cytokeratin-18 was found. There was evidence for marked expression of GrB by both CD8+ and CD4+ T-cells and natural killer cells in the respiratory tract. These findings suggest activation of the cell-mediated granzyme pathway during severe RSV-LRTI in children.
INTRODUCTION

Respiratory syncytial virus (RSV) is a major respiratory pathogen among infants and young children. Although in general RSV infection is limited to the upper respiratory tract the disease may progress to the lower airways leading to acute hypoxemic respiratory failure. The most severe cases of RSV-lower respiratory tract illness (RSV-LRTI) fulfil the criteria of acute lung injury (ALI) or the acute respiratory distress syndrome (ARDS). Despite decades of research the exact mechanisms that determine the development of severe RSV-LRTI in previously healthy children remain unclear.

One hypothesis proposes that activation of cell death pathways directed against RSV-infected cells and/or uninfected bystander cells contributes to disease severity. Although regulated cell death or apoptosis seems an important mechanism for RSV clearance, Welliver et al. showed marked expression of the apoptosis marker caspase-3 in airway epithelium of children with fatal RSV-LRTI, suggesting an imbalance in this process. Interestingly, increasing evidence implicates epithelial apoptosis in the pathogenesis of ALI/ARDS. Several studies demonstrated increased expression of classical apoptotic mediators such as FasL and granzymes in the lungs of adults with ALI/ARDS correlating with disease severity. These findings implicate enhanced activation of apoptotic pathways in the pathogenesis of acute inflammatory lung diseases and may also be relevant for severe RSV-LRTI in children.

In anti-viral host immune response an important cell death pathway is the granule exocytosis pathway involving the serine proteases granzyme (Gr) A and GrB, which is exploited by CD8+ T-lymphocytes (CTLs) and natural killer (NK) cells (reviewed elsewhere). Granzymes induce rapid cell death when directed into a target cell, and this mechanism is facilitated by the protein perforin, but may also occur independently from perforin. Detection of free extracellular GrA and GrB is considered to reflect cytotoxic activation of the cell-mediated immune response. However, proteolytic active extracellular granzymes may also contribute to the activation of pro-inflammatory cytokine release and degradation of the extracellular matrix, indicating that granzymes may be involved in several mechanisms of the host immune response.

Studies investigating the cell-mediated immune response in the lungs of children with RSV-LRTI have reported limited numbers of effector lymphocytes, although in rodents both CTLs and NK cells are recruited to the lungs upon primary RSV infection where they appear to contribute to disease pathogenesis. In the present study we hypothesized that severe RSV-LRTI in children is associated with local activation of the granzyme pathway by the cell-mediated host immune response. To test this, we investigated extracellular GrA and GrB and cellular expression of GrB in the respiratory tract of infants with RSV-LRTI.
METHODS

Patients and sample collection

All protocols were approved by the Academic Medical Center ethical committee and informed consent was obtained from parents. Tracheal aspirate (TA) samples were obtained from 23 children with RSV-LRTI and 12 age-matched controls without a pulmonary condition. All patients were admitted to the intensive care unit for mechanical ventilation (MV) between November 2003 and March 2006. Infection with RSV was proven by direct immunofluorescence assay (Imagen, DakoCytomation, UK) of nasopharyngeal aspirate. As part of an international randomized placebo-controlled trial on the use of glucocorticosteroids during RSV-LRTI (www.star-trial.com), RSV patients received dexamethasone 0.15 mg/kg/dose, intravenous or placebo (QID, 8 doses in total) starting within 24 h after start of MV. Oxygenation index calculated as (FiO₂ x mean airway pressure (cmH₂O) x 100)/ PaO₂ (kPa), during the first 24 h was used to assess severity of oxygenation anomaly. If more than one arterial blood gas was obtained, the best index was chosen. Pediatric risk of mortality (PRISM) scores were used as a measure of disease severity.

TA was collected as described before 18. Aspirate was collected without previous installation of fluids on the day of start of MV and in the RSV-patients on day 2 and 4 as long as the patient was intubated. An arterial blood sample was obtained from 15 RSV-patients on the day of start of MV.

Sample processing

After determination of the volume of the aspirate sample, an equal volume of cold 10 mM dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, MO) in 25 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) pH 8.0 was added at 4°C followed by 15 minutes mixing. If the aspirate remained mucoid this process was repeated once with a similar amount of DTT. Remaining cellular aggregates were cleared by adding DNAse (Sigma-Aldrich) at 4°C. Cells in processed aspirate were collected by 10 min centrifugation at 450 x g. Supernatant was aspirated, aliquoted and stored at -80°C. The cell pellet was resuspended in PBS and total white blood cell (WBC) were counted in a Bürker bright line counting chamber. Air dried cytospins were stained with Romanovsky (Diff-Quick) and differential WBC counts were obtained by counting 300 leukocytes using a standard light microscope. Reliable differential counts could not be obtained in 18% of the samples because of debris and some degeneration of cells.

Immunoassays

Extracellular GrA and GrB were measured by immunoassays (Sanquin, the Netherlands) according to the manufacturer’s description. Interleukin (IL)-8 was measured by immunoassay using monoclonal antibodies against IL-8 (R&D Systems) for capture and detection. Extracellular caspase-cleaved cytokeratin-18 (CK18), a marker of epithelial cell apoptosis, was
measured using the M30-Apoptosense sandwich immunoassay (Peviva AB, Sweden). The M30 antigen levels are expressed as Units/mL, where one Unit corresponds to 1.24 pmol of recombinant M30-containing peptide. Assay lower limit: 0.7 U/mL.

TA samples were diluted minimally 1:100 (GrA/B and IL-8) and 1:20 (cleaved CK18) to overcome interference of DTT with the assays and serial dilutions were tested. All reported antigen levels in TA are corrected for the sample processing dilution factor.

**Granzyme activity**

Activity of GrA was measured by a recently developed immunoassay as described elsewhere. For measurement of GrB activity, GrB in samples was captured on microtiter plates coated with anti-GrB mAb GB-11 (2 µg/ml in 0.1M sodium carbonate/bicarbonate buffer, pH 5.5). The plates were then washed and incubated with GrB-specific chromogenic substrate Ac-Ile-Glu-Thr-Asp-pNA (Alexis Biochemicals, Lausen, Switzerland; 0.4mM in 50mM Tris, 100mM NaCl and 0.1% Tween (v/v), pH 7.4). GrB activity was measured for 4 hours at 37°C at an absorbance (A) of 405nm on a Titer-Tek Multiscan (Labsystems, Helsinki, Finland) and is expressed as deltaA per hour.

**Fluorescent-activated cell sorting (FACS) analysis**

FACS analysis was performed in bronchoalveolar lavage fluid (BALF) samples from mechanically ventilated children with RSV-LRTI in the winters of 2006-2008. BALF was obtained by three subsequent instillations of 1 ml/kg of 0.9% saline through a wedged suction catheter passed through the endotracheal tube. After each instillation fluid was suctioned (mean ± SE recovery: 36% ± 4). The last 2 samples were pooled and 10 min centrifuged at 450 x g. The pooled BALF cells in FACS buffer (0.5% BSA, 0.02% potassium-EDTA in PBS) containing 10% normal human serum were labeled with APC-labeled anti-CD3, PerCP/Cy5-labeled CD8 or -CD4 (BD Pharmingen, San Jose, CA) and FITC-labeled anti CD16 (Sanquin, the Netherlands) and -56 (BD Pharmingen). For intracellular GrB staining cells were fixed and permeabilized with fixation/permeabilization solution (BD Pharmingen) and stained with PE-labeled anti-GrB mAb (Sanquin) or a PE-labeled isotype control (BD Pharmingen). Cells were analyzed using a FACSCalibur flow cytometer and CellQuest Pro software (BD Biosciences).

**Statistical analysis**

Not normally distributed data are expressed as medians and were analyzed by using the Mann-Whitney U test for differences between groups. For normally distributed data, a student’s t-test was used to compare group means. Spearman’s correlation coefficient was calculated to assess the degree of association between granzymes and studied markers. Proportions in the patient groups were compared by Fisher’s exact test. A two-sided p value of < 0.05 was considered statistically significant.
RESULTS

Baseline patient characteristics
The baseline characteristics of the patients are shown in Table 1. Ten RSV patients (43%) had received dexamethasone as part of the aforementioned randomized placebo-controlled trial. Total WBC counts and differentials in TA samples of controls and RSV patients are shown in Table 2.

<table>
<thead>
<tr>
<th></th>
<th>control*</th>
<th>RSV</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>12</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Age, months, median (IQR)</td>
<td>3.5 (6.6)</td>
<td>1.5 (1.0)</td>
<td>0.10</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>6 (50)</td>
<td>15 (65)</td>
<td>0.48</td>
</tr>
<tr>
<td>Duration of symptoms, days, median (IQR)</td>
<td>n.a.</td>
<td>3 (2)</td>
<td></td>
</tr>
<tr>
<td>PRISM score, median (IQR)</td>
<td>6 (6)**</td>
<td>10 (8)</td>
<td>0.09</td>
</tr>
<tr>
<td>Oxygenation index, median (IQR)</td>
<td>1.4 (0.5)§</td>
<td>4.4 (4.3)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Positive bacterial culture TA, n (%)¶</td>
<td>0 (0)</td>
<td>5 (22)</td>
<td>0.14</td>
</tr>
<tr>
<td>Duration of MV before 1st sample, hr, median (IQR)</td>
<td>9.5 (12.8)</td>
<td>19 (9)</td>
<td>0.15</td>
</tr>
</tbody>
</table>

* Reason for admission and mechanical ventilation of control patients were: metabolic disorder (n=1), intracardial rhabdomyoma (n=1), convulsions (n=1), pneumococcal meningitis (n=1), cardiomyopathy (n=1) and, postoperative (n=7: abdominal surgery (n=4), craniosynostosis (n=1), closure of persistent ductus arteriosis (n=1), congenital diaphragmatic hernia, (n=1)).
** (n=9) Of 3 controls no PRISM score was available at the time of TA sampling. § (n=8) No arterial blood gas analysis was obtained in 4 controls. ¶ On day of start of mechanical ventilation. MV: mechanical ventilation.

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>RSV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total WBC count (x 10⁶/ml), median (IQR)</td>
<td>6.8 (7.7)</td>
<td>28.6 (54.8)*</td>
</tr>
<tr>
<td>Differentials in %, median (range):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>81.4 (36.0-92.7)</td>
<td>88.5 (70.0-94.3)</td>
</tr>
<tr>
<td>Monocytes/macrophages</td>
<td>18.3 (7.3-64.0)</td>
<td>11.2 (5.0-30.0)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0 (0-2)</td>
<td>1.0 (0-2)</td>
</tr>
</tbody>
</table>

* p < 0.05, Mann-Whitney U test

Elevated GrA and GrB in TA during severe RSV-LRTI
The median (range) level of GrA in TA on the day of start of mechanical ventilation was 0.6 (0.3-14.3) ng/ml and 11.1 (0.3-98.4) ng/ml in controls and RSV-patients respectively (p<0.01, Figure 1A). Likewise, the median (range) level of GrB in TA was higher in the RSV patients (69.0, 3.1-728.0, ng/ml) as compared to the controls (1.7 ng/ml, 0.5-39.6, ng/ml) (p < 0.01, Figure...
There was a significant correlation between the levels of GrA and GrB in TA (Spearman $r = 0.67$, $p<0.001$). In the RSV patients, the plasma levels of GrA (median 63.0, range 3.0-180.0, pg/ml) and GrB (median 35.0, range 13.0-92.0, pg/ml) on the first day of MV were significantly lower than the levels in TA ($p<0.001$ for both comparisons, data not shown).

GrA and GrB in TA were detected throughout the course of RSV-LRTI, but no effect of dexamethasone as compared with placebo on the granzyme levels was found (Figure 1C and D). No difference in granzyme levels was found between RSV patients with or without a positive bacterial culture of TA.

GrA and GrB in TA have retained activity

To ascertain biological activity of GrA and GrB in the airways of RSV patients we measured active GrA and GrB with enzyme capture assays. Because the sensitivity of these assays was 50 pg/ml and 1 ng/ml respectively, only 10 samples of TA containing high levels of GrA and

**Figure 1.** A-B, levels of GrA and GrB (ng/ml) in TA from children without pulmonary condition (controls, n=12) or with RSV-LRTI (n=23) on the day of start of mechanical ventilation (MV). Bars represent median values. Dotted lines represent lowest detectable levels. * $p < 0.01$. C-D, relative change of median levels of GrA and GrB in TA on day 2 (n = 23) and day 4 (n = 14) from baseline (day of start of MV, n=23) in RSV patients who received placebo (□, n=13) or dexamethasone (■, n=10) treatment. Error bars show the 25th or 75th percentile. $p = n.s.$
GrB were measured. There was a significant correlation between the levels of active GrA and total GrA antigen (Spearman $r = 0.82, p < 0.01$) (Figure 2A). In addition, we found a positive correlation between GrB activity and total GrB antigen (Spearman $r = 0.62, p = 0.05$) (Figure 2B).

**Granzymes in TA correlate with inflammatory markers, but not with cleaved-CK18**

There was a positive correlation between GrA and GrB and total WBC counts and IL-8 in TA in the RSV patients and this correlation tended to be stronger after the first days of MV (Figure 3). However, no correlation between granzyme levels and disease severity at baseline, as determined by PRISM scores and oxygenation index, was found (data not shown).

To study lung epithelial apoptosis in relation to extracellular granzymes, we analyzed the correlation between GrB and cleaved-CK18 in TA. Cytokeratin-18 is an epithelium-specific intermediate filament protein which is cleaved by caspases early during apoptosis.

**Table 3.** Mean percentages ± SE (of total cells) of cells expressing CD3, CD3+CD8 and CD3-CD16/56 (NK cells) in BALF of mechanically ventilated children with severe RSV infection, as determined by FACS analysis.

<table>
<thead>
<tr>
<th>Day</th>
<th>CD3 % of total cells</th>
<th>CD8 % of total cells</th>
<th>CD3-CD16/56 % of total cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>start of MV</td>
<td>1.7 ± 0.8</td>
<td>0.6 ± 0.3</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>1.6 ± 0.7</td>
<td>0.9 ± 0.4</td>
<td>1.1 ± 0.7</td>
</tr>
<tr>
<td>4</td>
<td>2.1 ± 0.8</td>
<td>1.3 ± 0.5</td>
<td>0.8 ± 0.1</td>
</tr>
</tbody>
</table>

MV: mechanical ventilation. First day of MV: n = 7, day 2: n = 6, day 4: n = 4.
and may be subsequently released from cells into the extracellular space \(20\). Caspases are activated by GrB, but not by GrA. There was no significant correlation between GrB and cleaved-CK18 in TA at any time point. Also, no statistically significant difference between cleaved-CK18 levels (U/mL) of controls (median 14.5, IQR 39.2) as compared to the RSV patients (median 8.4, IQR 17.7) was found (p=0.20).

**GrB expression by lymphocytes in BALF**

In order to find a potential source of granzymes in the lungs of children with severe RSV-LRTI, we performed FACS analysis in BALF samples in an additional cohort of mechanically ventilated children (mean age 1.4 ± 0.2 months, n=7) with RSV-LRTI. These patients did not differ from the original cohort in terms of age, gender distribution and duration of illness (data not shown). T-cells and NK cells were detected in low numbers throughout the course of RSV-LRTI (Table 3). GrB-positive cells were found predominantly among cells within the side/forward scatter region of the lymphocyte population (Figure 4A-E). In addition to CD8+ T-cells and NK cells, CD8- T-cells were found to express GrB (Figure 4F-J). This latter observation suggests that besides CD4-CD8- T-cells (which may include gamma/delta T-cells) CD4+ T-cells may express GrB, which was confirmed in an additional FACS analysis of 4 RSV patients (Figure 4K). The percentages of GrB-positive T-cells and NK cells and corresponding mean fluorescence intensity are shown in Figure 5.

![Figure 3](image_url)

**Figure 3.** A, correlation between the levels of GrA (●) or GrB (○) and total white blood cell counts (A) and IL-8 levels (B) in TA of RSV patients on day 4 after start of MV. Spearman \(r > 0.63\) (p<0.05) for all comparisons.
Figure 4. Representative examples of FACS scatter plots of RSV patients. A, Side/anti-GrB mAb scatter plot showing the GrB-positive cell population (arrow), which is not detected in the same sample stained with an isotype control for GrB (B). C, Side/forward scatter plot of the gated GrB-positive cell population. D, Side/anti-CD3 mAb scatter plot showing a CD3-positive population (arrow). E, Side/forward scatter plot of the gated CD3-positive cell population. Note that the GrB-positive cell population (see C) is detected in the same side/forward region as the CD3-positive cell population. F, anti-CD3/anti-CD8 mAb scatter plot of a gated lymphocyte region (see E) showing CD3⁺CD8⁻ cells (*) and CD3⁺CD8⁺ cells (**). G, anti-CD3/anti-CD16/56 mAb scatter plot of a gated lymphocyte region (see E) showing CD3⁺CD16/56⁺ (NK) cells. H, anti-GrB/anti-CD8 mAb scatter plot of a gated CD3⁺CD8⁺ cell population (I, corresponding GrB isotype control plot). I, anti-GrB/anti-CD3 mAb plot of a gated CD3⁺CD8⁺ cell population (M, corresponding GrB isotype control plot). J, anti-GrB/anti-CD16/56 mAb plot of a gated CD3⁺CD16/56⁺ cell population (N, corresponding GrB isotype control plot). K, anti-GrB/anti-CD4 mAb plot of a gated CD3⁺CD4⁺ cell population (O, corresponding GrB isotype control plot).
DISCUSSION

The main goal of this study was to determine whether severe RSV infection in children is associated with local activation of the granzyme pathway by the cell-mediated host immune response. We report high levels of proteolytic active extracellular GrA and GrB and marked expression of GrB by T-lymphocytes and NK cells in the respiratory tract of children during the course of severe RSV-LRTI. The levels of extracellular GrA and GrB correlated with total WBC counts and IL-8 levels in the airways after the acute-onset of RSV disease, but no association with the epithelium apoptosis marker cleaved-CK18 could be demonstrated.

The present study extends our understanding of cell-mediated cytotoxic responses during severe RSV-LRTI in children. Numerous rodent studies have shown that both NK cells and CTLs are recruited to the lungs during primary RSV infection. Graham et al. reported that although CTLs are involved in the clearance of RSV, depletion of these lymphocytes diminishes clinical illness upon exposure to RSV. Treatment of RSV-infected CTL depleted mice with high dose RSV-specific CTLs results in viral clearance, but augments lung injury by causing severe haemorrhage and neutrophilic infiltration. These findings suggest that cell-mediated cytotoxic responses play an important role in RSV disease pathogenesis in mice, but the relevance in humans is unclear. Studies in vitro show that CTLs isolated from peripheral blood of RSV infected infants lyse infected autologous target cells. However, Welliver et al. recently reported the near absence of CTLs and NK cells in lung tissues from 9 infants who died of RSV-LRTI. Previous analysis of BALF samples of RSV-infected children showed that a small percentage of the total cells in the lungs is CD8-positive. Similarly, in the present study we found low

Figure 5. Mean percentages (A) and mean fluorescence intensity (B) of GrB-positive CTLs (CD3+CD8+), CD3+CD8+ cells and NK cells (CD3+CD16/56+) in BALF throughout the course of severe RSV-LRTI. First day of mechanical ventilation: n = 7, day 2: n = 6, day 4: n = 4. Error bars show the standard error.
numbers of T-lymphocytes and NK cells in the lungs. However, despite these low cell counts, we report high levels of extracellular granzymes and marked GrB expression in these cell populations in the respiratory tract.

The activation of the cell-mediated granzyme response in children with severe RSV-LRTI raises the possibility that this cell death pathway is involved in RSV disease pathogenesis. Direct killing of infected cells by granzymes is considered a major host defence mechanism against viruses in general. In mice, both GrA and GrB are essential in controlling ectromelia and cytomegalovirus infection. Interestingly, viruses have been reported to encode proteins that inhibit granzymes, suggesting pathogen immune evasive adaptations. On the other hand enhanced death of infected cells and/or bystander cells may contribute to the development of disease by causing tissue dysfunction as has been suggested for severe inflammatory lung diseases. Welliver et al. found marked expression of caspase-3 in airway epithelium of children with fatal RSV-LRTI, a finding consistent with studies performed in adult patients with ALI/ARDS. The study of Welliver et al. suggests an imbalance in apoptosis during severe RSV-LRTI, but the involved cell death pathways remain unclear. Our findings may point toward a role for the granzyme pathway in apoptotic cell death during severe RSV-LRTI, but contrary to our expectations, we found no correlation between the levels of extracellular GrB and caspase-cleaved CK18 in the airways. The release of caspase cleaved CK18 is a surrogate marker of epithelial apoptosis but has not been studied extensively in vivo. The detection of cleaved CK18 in serum was found to correlate well with tissue damage in patients with hantavirus infection, but it is unclear how this protein behaves and reflects epithelial injury in other body fluids, including BALF. Histological analysis of apoptosis to confirm our data is highly preferred but could not be performed in our study for obvious ethical reasons.

In addition to CTLs and NK cells, CD4+ T-cells and basophils may express granzymes. Recently, neutrophils also were shown to express granzymes, although this has been debated by others. In the present study, GrB-positive cells were only found among lymphocytes. CTLs seemed to contribute to GrB expression the most, but actual degranulation and subsequent granzyme release may have differed between the studied cell populations. The highest GrB expression by NK cells occurred on the first day of admission, before the peak of GrB expression by T-cells. Similarly, in RSV infected mice the cytotoxic activity of NK cells is highest before CTLs are recruited to the lungs. Remarkably, granzyme levels in the airways during severe RSV-LRTI were not affected by treatment with dexamethasone, although several studies in vitro have shown suppression of CTL and NK cytotoxic activity by corticosteroids.

Our finding of GrB expression by CD4+ T-lymphocytes is interesting because it suggests that the granzyme pathway may be utilized to modulate immune responses to RSV. Devadas et al. have shown that Th2 cells express GrB which induces their own cell death. Furthermore, they reported that GrB deficient mice have augmented Th2 cytokine
responses and lung cellular infiltrations in a model of allergic inflammation. Grossman et al. have shown that the granzyme pathway is exploited by natural T-regulatory cells (CD4+CD25+) against autologous activated CD8+ and CD4+ T-cells. These findings suggest that granzymes function to control immune responses, in addition to their role in direct virus-infected cell killing. In contrast, another line of research proposes that granzymes actively participate in the activation of pro-inflammatory responses. For example, studies in vitro have shown that proteolytic active GrA induces the release of IL-6 and IL-8 from epithelial cells and lung fibroblasts. In the present study, granzyme levels significantly correlated with IL-8 levels in the airways during severe RSV-LRTI and with total WBC counts after the acute phase of respiratory failure. Moreover, we found evidence that active forms of GrA as well as GrB were present, confirming the potential to exert a biological function.

This study has three potential limitations. First, all patients had severe acute hypoxemic respiratory failure necessitating MV and thus represent the end of the spectrum of RSV disease. This withholds us from drawing firm conclusions when relating the extent of activation of the granzyme pathway with markers of disease severity such as PRISM scores and oxygenation index. Second, because all patients (including controls) were subjected to MV and supplemental oxygen it is possible that this might have had (synergistic) effects on granzyme levels, inflammation or apoptosis. Third, soluble granzymes were measured in TA that may reflect more localized (and thus variable) proximal epithelial lining fluid.

In conclusion, severe RSV-LRTI in children is associated with high levels of proteolytic active extracellular GrA and GrB and expression of GrB by lymphocytes in the respiratory tract. These findings suggest that the granzyme pathway is activated by the local cell-mediated host immune response to RSV. Further studies should elucidate the exact role of granzymes in the lungs during RSV disease.
REFERENCES


Granzyme A and B-cluster Deficiency Delays Acute Lung Injury in Pneumovirus-Infected Mice

Reinout A. Bem¹
Job B.M. van Woensel¹
Rene Lutter²
Joseph B. Domachowske³
Jan Paul Medema⁴
Helene F. Rosenberg⁵
Albert P. Bos¹

¹ Pediatric Intensive Care Unit, Emma Children’s Hospital AMC, Amsterdam, The Netherlands; ² Departments of Pulmonology and Experimental Immunology, Academic Medical Center, Amsterdam, The Netherlands; ³ Department of Pediatrics, Upstate Medical University, Syracuse, NY, USA; ⁴ Laboratory for Experimental Oncology and Radiobiology, Academic Medical Center, Amsterdam, The Netherlands; ⁵ Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA

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ABSTRACT

Lower respiratory tract infection by the human pneumovirus respiratory syncytial virus is a frequent cause of acute lung injury in children. Severe pneumovirus disease in humans is associated with activation of the granzyme pathway by effector lymphocytes, which may promote pathology by exaggerating pro-apoptotic caspase activity and pro-inflammatory activity. The main goal of this study was to determine whether granzymes contribute to the development of acute lung injury in pneumovirus-infected mice. Granzyme-expressing mice and granzyme A, and B-cluster single and double-gene deleted mice were inoculated with the rodent pneumovirus pneumonia virus of mice strain J3666, and were studied for markers of lung inflammation and injury. Expression of granzyme A and B is detected in effector lymphocytes in mouse lungs in response to pneumovirus infection. Mice deficient for granzyme A and the granzyme B-cluster have unchanged virus titers in the lungs, but show a significantly delayed clinical response to fatal pneumovirus infection, a feature that was associated with delayed neutrophil recruitment, diminished activation of caspase-3 and reduced lung permeability. We conclude that granzyme A and B-cluster deficiency delays the acute progression of pneumovirus disease by reducing alveolar injury.
INTRODUCTION

The human pneumovirus, respiratory syncytial virus (RSV), is the leading cause of lower respiratory tract illness in young children and infants worldwide. In Western countries, the annual incidence rate of RSV-associated hospitalization may be as high as 30 per 1000 children under 1 year of age. RSV disease in these children presents as bronchiolitis or pneumonia. However, the severe end of the RSV disease spectrum includes the development of acute lung injury and acute respiratory distress syndrome (ALI/ARDS), which is characterized by severe hypoxemia and bilateral lung infiltrates in the setting of a normal cardiac preload. Currently, treatment for severe RSV disease in normal healthy children is limited to supportive measures, stressing the need to further explore its pathogenesis.

In general, ALI/ARDS is associated with the development of diffuse neutrophilic alveolitis and loss of alveolar capillary barrier integrity as a result of enhanced lung epithelial cell death. Interestingly, also in humans with severe RSV disease a large number of neutrophils is recruited to the lungs, and caspase-dependent apoptosis of lung epithelial cells is observed. Similarly, in the pneumonia virus of mice (PVM) mouse model for severe RSV disease, pulmonary neutrophil recruitment and caspase-3 activation is associated with enhanced lung permeability and clinical signs of illness. These findings suggest that the coordinated actions of pro-inflammatory and pro-apoptotic mediators may serve to augment the pathologic responses characteristic of severe pneumovirus disease, although the nature of the mediators linking these responses are not yet fully understood.

Granzymes (Gzms) are effector molecules that may contribute to both inflammatory and apoptotic features of RSV disease. Gzms are serine proteases located in granules of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells and have traditionally been considered to be crucial to the anti-viral immune response. Gzms are delivered into the cytosol of target cells by the pore-forming protein perforin, but intracellular trafficking is also known to be facilitated by adenovirus and bacterial toxins. Of the five Gzms found in humans (GzmA, B, H, K and M), the tryptase GzmA and aspartase GzmB are the most thoroughly characterized. It has been shown that GzmB induces classical (caspase-dependent) apoptosis in target cells, and in vitro micromolar concentrations of GzmA, in the presence of perforin, can induce caspase-independent DNA damage. However, Metkar at al. reported recently that GzmA-mediated cytotoxicity at reduced, more physiologic concentrations may be relatively low. Instead, active soluble GzmA at lower concentrations induces the release of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α from monocyte/macrophage, fibroblast and epithelial cell lines via a perforin-independent mechanism. This raises the possibility that Gzms have a broad functionality in the human immune response to viral infection in vivo, including activation of (caspase-dependent) cell death and inflammation.
Recently, our group has demonstrated that tracheal aspirates from mechanically-ventilated children with severe RSV infection contain high levels of biologically active soluble GzmA and GzmB. To investigate whether these Gzms may actively participate in the pathogenesis of RSV disease, specifically promoting progression to ALI/ARDS by inducing pro-inflammatory and caspase-dependent pro-apoptotic activity, we explored lung inflammatory and injury markers in acute pneumovirus infection in GzmA and/or GzmB-cluster gene deleted mice.

**METHODS**

**Virus and mouse strains**

The fully pathogenic PVM strain J3666 was originally obtained from Dr. A.J. Easton (University of Warwick, Coventry, U.K.) from virus stocks originating at the Rockefeller University. Virulence was maintained by continuous passage in mice. Virus titer in stock aliquots was $12 \times 10^7$ copies of PVM/ml, as determined by quantitative PCR (see below), and PVM inocula per mouse were as indicated.

Nine- to twelve-week-old female mice were used in all experiments. C57Bl/6 wild type mice (CD45.1+) were originally obtained from Jackson Laboratories (Bar Harbor, ME, USA) and bred in house. The GzmA−/−, GzmB−/− (generated by the group of Dr. T. Ley) and GzmA−/−GzmB−/− mice on a C57Bl/6 background were originally obtained from Dr. M.M. Simon and bred in house. GzmA+/−GzmB+/− were used to serve as a primary positive control for the GzmA−/−GzmB−/− mice: GzmA+/−GzmB+/− mice were generated by crossing GzmA−/− with GzmB−/− mice resulting in a similar mixed C57Bl/6 substrain (B6J and B6-III) background as the GzmA−/−GzmB−/− mice. Importantly, the GzmB−/− (and GzmA−/−GzmB−/−) mice lack the entire GzmB-cluster which includes GzmC, -D and -F due to knockdown by a retained phosphoglycerate kinase I gene promoter (PGK-neo) cassette.

**Animal protocols**

The animal protocols as described below were approved by the Animal Care and Experimental Research Committee of the Academic Medical Center Amsterdam, the Netherlands.

PVM diluted in Roswell Park Memorial Institute (RPMI)-medium (Invitrogen Ltd, Paisley, UK) in a total volume of 80µl was delivered via intranasal inhalation to isoflurane anesthetized mice. At day 3, 7 or 8 after inoculation the mice were euthanized with intraperitoneal pentobarbital (120mg/kg), the left lung was removed and flash-frozen in liquid nitrogen for homogenization. Bronchoalveolar lavage (BAL) was performed in the right lung by instilling four separate 0.5 ml aliquots of 0.9% NaCl containing 0.6 mM EDTA. The BAL fluid (BALF) was maintained at 4°C, and spun at 200 g for 10 min.
The supernatants were stored at -80ºC as individual aliquots, and the cell pellet was processed for total cell counts and differentials. For histological analysis on day 8 after PVM inoculation the right lung was fixed with 10% formalin immediately after the BAL procedure.

**Measurements**

**Microarray and flow cytometry analysis.** The microarray study as described previously, was probed to determine the expression profile of GzmA and GzmB transcripts in whole lung tissue in response to PVM infection. To determine intracellular expression of GzmA and GzmB in effector lymphocytes, BALF leukocytes in FACS buffer (0.5% BSA, 0.02% potassium-EDTA in PBS) were probed with FITC-labeled anti-CD8, PerCP-Cy5-labeled anti-NK1.1 and APC-labeled anti-CD3 (all anti-mouse from eBioscience, San Diego, CA, USA). Next, cells were fixed and permeabilized with fixation/permeabilization solution (BD Biosciences, Franklin Lakes, NJ) and stained with PE-labeled mouse anti-mouse GzmA mAb (Santa Cruz Biotechnology Inc., Heidelberg, Germany) or with PE-labeled rat anti-mouse GzmB mAb (eBioscience) in 10% normal rat serum to block non-specific binding. The FACS-Calibur flow cytometer (BD Biosciences) and FlowJo software (Tree Star Inc, Ashland, OR) were used for analysis.

**Lung virus titers.** Lungs were homogenized in Trizol reagent to extract RNA, according to the manufacturer’s description (Invitrogen Ltd., Paisley, UK). RNA was resuspended in DEPC-treated water. After DNasel treatment of 2 µg RNA (Applied Biosystems, Foster City, CA) cDNA synthesis was performed using a random hexamer cDNA synthesis kit (Applied Biosystems). Copies of the PVM sh gene (GenBank: AY573815) were detected in quantitative real-time PCR reactions containing 1 µl cDNA, Taqman PCR Master Mix (Applied Biosystems), 77 nM TAMRA probe (5’-6FAM-CGCTGATAATGGCCTGCAGCA TAMRA-3’), 200 nM primers (5’-GCCTGCATCAACACAGTGTGT-3’ and 5’-GCCTGATGTGGCAGTGCTT-3’). The gapdh housekeeping gene was detected in cDNA samples using rodent gapdh primers (100nM) and VIC-probe (200nM) (Ambion, Applied Biosystems). Standard curves with known concentrations of the full-length sh-gene and gapdh decatemplate (Applied Biosystems) were used for quantification. Results are expressed as copies of PVM-sh per 10^9 copies of gapdh.

**Lung inflammatory markers.** Total BALF leukocyte counts were performed on an aliquot of the BALF cells using a Bürker bright line counting chamber. Cytospins were stained with Romanovsky (Diff-Quick) and differential WBC counts were obtained by counting 200 leukocytes using a standard light microscope. Lung cytokines in BALF were measured by multiplex fluorescent bead assay for TNFα, IFNγ, IL-6, IL-1β, KC and MIP-2 (Luminex, R&D systems, Minneapolis, MN).

**Lung caspase-activation.** Caspase-3 immunohistochemistry on 5-µm-thick paraffin embedded lung sections was performed using rabbit anti-cleaved (active) caspase-3 mAb (Cell Signaling, Danvers, MA) followed by secondary labelling with anti-rabbit IgG HRP.
polymer (Immunologic, Duiven, The Netherlands). HRP activity was detected with Peroxidase Substrate Kit (Vector® NovaRED, Vector Laboratories, Burlingame, CA). Quantification of the overall lung caspase-3 activity in lung homogenates corrected for lung weight was performed using the caspase-3/CPP32 Fluorometric Assay kit from Biovision, Mountain View (CA) as described previously.

**Lung permeability.** Leakage of the high molecular weight serum protein $\alpha$-macroglobulin or IgM into the lungs was measured by immunoassay for mouse $\alpha$-macroglobulin (Life Diagnostics, West Chester, PA) or mouse IgM (Bethyl Laboratories, West Chester, PA) performed on BALF.

**Lung histology.** A blinded histology analysis for evidence of lung injury was performed on haematoxylin and eosin stained lung sections. A pathological grade for each lung section was determined according to the following criteria: 1 = presence of areas with cellular alveolitis involving 25% or less of the lung parenchyma; 2 = lesions involving 25–50% of the lung; 3 = lesions involving 50% or more of the lung. An additional point was given when there were alveolar wall alterations such as thickening or capillary congestion outside the aforementioned inflammatory areas.

**Clinical response.** Total body weight and a clinical scoring system previously validated in multiple PVM mouse studies was used: score 1 = healthy, no signs of illness, 2 = subtle ruffled fur, 3 = evident ruffled fur with hunched posture, 4 = evident lethargy with abnormal breathing pattern, 5 = moribund, 6 = death; modified from Cook et al. 30. The endpoint for sacrifice used in this study was a score of 5 and/or loss > 20% of starting body weight.

**Statistical analysis**
To detect differences among groups we performed either unpaired t-test (for comparison of two groups) or one-way ANOVA (for comparison of multiple groups) followed by LSD post hoc analysis on log10 transformed data. A p-value of < 0.05 was considered statistical significant.

**RESULTS**

**Expression of GzmA and GzmB in effector lymphocytes in lungs of PVM-infected mice**
Severe pneumovirus (RSV) disease in children is associated with increased local levels of biologically active GzmA and GzmB, and cellular expression of GzmB predominantly in BALF CTLs 20. To determine whether infection with the murine pneumovirus PVM induces GzmA and GzmB expression in mice we analyzed the temporal gene expression profiles of transcripts encoding GzmA and GzmB from a microarray study performed on total lung RNA
from C57Bl/6 mice inoculated with a non-lethal dose of PVM. Transcripts encoding both GzmA and GzmB were detected in lung tissue from PVM infected mice (Figure 1A). Levels of both the GzmA and GzmB transcript reached a peak at day 7 and remained above baseline levels until day 14 after inoculation. Immunoreactive GzmA was detected predominantly in CD3−/NK1.1+ (NK) cells, and GzmB in CD8+/CD3+ (CTL) cells isolated from BALF at day 7 of infection (Figure 1B).

PVM infection in GzmA and GzmB-cluster gene deleted mice

Based on the Gzm gene expression profile, we hypothesized that if the Gzm response contributes either positively or negatively to severe pneumovirus disease, this would become apparent at or beyond peak expression at day 7. To address this question we investigated lung virus titers, caspase-3 activation and cytokine release in wild type, GzmA or GzmB single-gene deleted and GzmAGzmB double-gene deleted mice on day 7 and day 8 after inoculation with PVM (Figure 2).

Surprisingly, whole lung virus titers from PVM-infected wild type mice were indistinguishable from those measured in lungs from PVM-infected GzmA−/−, GzmB−/− and GzmA−/−GzmB−/− mice, both on day 7 and 8 after inoculation as determined by quantitative RT-PCR (Figure 2A). However, caspase-3 activity in lung homogenates of the GzmA−/−GzmB−/− mice was significantly reduced compared to the wild type and to the GzmA−/− mice on day 7 and 8 after PVM inoculation (Figure 2B). Previously we have identified that reduced whole lung caspase-3 activity is associated with decreased leakage of the high molecular...
weight serum protein \(\alpha\)-macroglobulin into the alveolar spaces in severe PVM disease in mice. In line with this, GzmB\(^{-/-}\) and GzmA\(^{-/-}\) GzmB\(^{-/-}\) mice had reduced concentrations of \(\alpha\)-macroglobulin in BALF, as compared to wild type and GzmA\(^{-/-}\) mice, although this reached statistical significance only for the GzmB\(^{-/-}\) mice (Figure 2C).

PVM infection in mice promotes robust neutrophil influx into the lungs. Neutrophil recruitment, as determined by BALF total neutrophil counts, was not different among the wild type, GzmA\(^{-/-}\), and GzmB\(^{-/-}\) mice; in contrast neutrophil recruitment was delayed in the GzmA and the GzmB-cluster double-gene deleted mice (Figure 2D). However, this delay was not associated with the release of the pro-inflammatory cytokines IL-1\(\beta\) (not detected in any of the mice), IL-6 (Figure 2E) and TNF\(\alpha\) (Figure 2F) in the lungs.

**PVM infection in GzmA\(^{-/-}\)GzmB\(^{-/-}\) and GzmA\(^{+/+}\)GzmB\(^{+/+}\) mice**

The findings thus far suggest that Gzms may alter the response to PVM, including caspase-mediated apoptosis and lung permeability, but do not affect viral clearance or the release of the pro-inflammatory cytokines IL-1\(\beta\), IL-6 and TNF\(\alpha\). We found a more pronounced phenotype in the double-gene deleted mice consistent with previous findings. As such, we proceeded with a focus on the GzmA\(^{-/-}\)GzmB\(^{-/-}\) mice, and to account for the differences in the genetic background of the substrains that may have affected the results above, we...
**Figure 3.**

A, virus titer in the lung, expressed as number of PVM-sh copies per $10^9$ gapdh copies; B, lung homogenate caspase-3 activity (arbitrary fluorescence units), in the GzmA$^{+/-}$/GzmB$^{+/-}$ mice and GzmA$^{+/-}$/GzmB$^{-/-}$ mice on day 7 (n=6 per group) and 8 (n=6 per group) after PVM inoculation ($6 \times 10^3$ copies). * p<0.05. Data are shown as box plots depicting the median, interquartile range and full range. C, representative images of cleaved caspase-3 immunohistochemistry in lung tissues of the GzmA$^{+/-}$/GzmB$^{+/-}$ mice and GzmA$^{+/-}$/GzmB$^{-/-}$ mice on day 8 after PVM inoculation. Note the relative positive staining in alveolar wall cells (arrows) as compared to bronchial epithelial cells in the GzmA$^{+/-}$/GzmB$^{+/-}$ mice.
compared the lung responses to PVM infection of GzmA<sup>−/−</sup>GzmB<sup>−/−</sup> mice to GzmA<sup>+/−</sup>GzmB<sup>+/−</sup> mice, which are on the same mixed C57Bl/6 substrain (B6J and B6-III) background.<sup>22;25</sup>

**Viral titers and caspase activation.** Consistent with our findings above, whole lung virus titers remained indistinguishable (Figure 3A), while there was a marked reduction in caspase-3 activity in lung homogenates in the GzmA<sup>−/−</sup>GzmB<sup>−/−</sup> mice as compared to the GzmA<sup>+/−</sup>GzmB<sup>+/−</sup> mice (Figure 3B). To investigate the lung tissue distribution of active caspase-3 in the mice, we performed immunohistochemical localization for cleaved caspase-3 in lung tissue sections of day 8 after PVM inoculation. The lung tissues of GzmA<sup>+/−</sup>GzmB<sup>+/−</sup> mice showed widespread cleaved caspase-3 staining in alveolar wall cells and among infiltrating leukocytes (Figure 3C). Interestingly, although PVM replication is detected primarily in bronchial epithelial cells,<sup>27</sup> cleaved caspase-3 was not evident in these cells.

**Lung permeability.** As compared to the GzmA<sup>+/−</sup>GzmB<sup>+/−</sup> mice, the GzmA<sup>−/−</sup>GzmB<sup>−/−</sup> mice displayed diminished concentrations of α-macroglobulin and IgM in BALF, both markers for lung permeability<sup>9</sup> (Figure 4A and B).

![Figure 4](image.png)

**Figure 4.** Concentration of α-macroglobulin (A) and IgM (B) in BALF of the GzmA<sup>+/−</sup>GzmB<sup>+/−</sup> mice and GzmA<sup>−/−</sup>GzmB<sup>−/−</sup> mice on day 7 (n=6 per group) and 8 (n=6 per group) after PVM inoculation (6 x 10<sup>3</sup> copies). * p<0.05. Data are shown as box plots depicting the median, interquartile range and full range.

**Lung inflammation.** As compared to the GzmA<sup>+/−</sup>GzmB<sup>+/−</sup> mice, the GzmA<sup>−/−</sup>GzmB<sup>−/−</sup> mice had decreased total neutrophil counts in BALF on day 7 after PVM inoculation (Figure 5A). However, this apparent delay in neutrophil influx was not associated with the release of cytokines IL-1<sub>β</sub> (not detected in any of the mice), IL-6 (Figure 5B), TNFα (Figure 5C), MIP-2 (Figure 5E) or KC (Figure 5F). IFNγ is among the critical regulators of the neutrophil response in PVM infection,<sup>32</sup> and was the only cytokine that correlated with the delay in neutrophil recruitment in the GzmA<sup>−/−</sup>GzmB<sup>−/−</sup> mice (Figure 5D).

**Lung histology.** On day 8 after inoculation with PVM, the lung tissues from all mice showed extensive peri-bronchial and peri-vascular cellular infiltrates, as well as areas with acute alveolitis and alveolar wall alterations such as thickening and (mild hemorrhagic)
capillary congestion, similar to findings described previously. However, cellular alveolitis and capillary congestion was more diffuse in the GzmA+/−GzmB+/− mice, when compared to GzmA−/−GzmB−/− mice (Figure 6): mean (± SE) histopathology scores were 3.2 (0.3) in the GzmA+/−GzmB+/− mice, and 2.2 (0.2) in the GzmA−/−GzmB−/− mice (p<0.05).

Figure 5. Total neutrophil (PMN) counts (A), and concentrations of IL-6 (B), TNFα (C), IFNγ (D), MIP-2 (E) and KC (F) in BALF of the GzmA+/−GzmB+/− mice and GzmA−/−GzmB−/− mice on day 7 (n=6 per group) and 8 (n=6 per group) after PVM inoculation (6 x 10^3 copies). * p<0.05. Data are shown as box plots depicting the median, interquartile range and full range.
Clinical response. Finally, to address whether these Gzm-mediated alterations of the lung response to PVM affect the clinical phenotype, we followed clinical scores and body weight up to day 12 after inoculation with a lethal inoculum of PVM. The GzmA+/GzmB+/− mice showed a statistically significant delay in reaching the maximum clinical response to PVM (Figure 7A). All the GzmA+/GzmB+/− mice reached the defined endpoint (clinical score of 5 and/or body weight loss > 20%) at day 10 after PVM inoculation, whereas at this point this was reached by 67% of the GzmA−/GzmB−/− mice (p<0.05). A delayed response to PVM infection among the GzmA−/GzmB−/− mice is also observed when weight loss alone as a sole, purely objective measure of the clinical response was examined (Figure 7B).
DISCUSSION

The primary goal of this study was to determine whether Gzms promote pathophysiologic responses, including acute lung injury, in pneumovirus-infected mice. In this work, we detect both GzmA (predominantly in NK cells) and GzmB (predominantly in CTLs) in association with severe respiratory sequelae induced by the mouse pneumovirus pathogen, PVM. We found that mice deficient for both GzmA and the GzmB-cluster (GzmA−/− GzmB−/−) have a delayed clinical response to fatal PVM infection, although Gzm expression has no impact on virus replication or clearance per se. Instead, we determine that the absence of Gzms results in delayed neutrophil recruitment, diminished activation of caspase-3 and reduced lung permeability.

The Gzm system exploited by effector lymphocytes plays a major role in host anti-viral immunity by activating cell death programs in infected target cells. Providing the first in vivo evidence for Gzm-mediated viral elimination, Mullbacher et al. showed that the survival and clearance of the mouse poxvirus, ectromelia, in mice lacking both GzmA and the GzmB-cluster was markedly depressed as compared to wild type mice. Systematic challenge of Gzm-deficient mice to different virus families has not been undertaken, but replication of cytomegalovirus and herpes simplex virus have also found to be controlled by Gzm activity. At the same time, there is much evidence that effector lymphocyte-mediated immunopathogenic mechanisms form the basis of enhanced disease in certain virus infections, and this includes the development of severe human pneumovirus (RSV) disease. For example, both CTLs and NK cells are recruited to the lungs during primary RSV infection in mice. Depletion of CTLs in mice reduces clinical signs of RSV disease, despite delaying viral clearance. While intravenous transfer of high dose CTLs into CTL-depleted mice causes severe hemorrhage and neutrophilic inflammation. Frey et al. studied the PVM mouse model, which, unlike RSV infection in mice, reproduces several features of severe RSV disease in humans, including robust viral replication, pro-inflammatory alterations in the airways and alveoli, alveolar injury and importantly, overt clinical signs of illness. They found similar results, namely, that T-cell deficient mice have a delayed clearance of PVM, but reduced pulmonary inflammatory infiltrates and weight loss. These findings suggested that effector lymphocytes may contribute to the immunopathophysiology associated with human RSV infection, but whether the Gzm system is involved herein remained unclear from those studies.

Recently, we found high levels of active GzmA and GzmB in tracheal aspirates from children with severe RSV disease. Surprisingly, in the present study, virus titers in lung tissue were not altered by Gzm-targeting, despite a major reduction in lung caspase-3 activation in GzmA and GzmB-cluster deficient mice. Similarly, Frey et al. found that mice deficient for perforin, which facilitates granzyme-trafficking into virus-infected target cells, did not alter PVM lung titers. These findings suggest that alternative pathways may play more prominent roles in PVM clearance. Certain viruses, such as adenovirus and...
parainfluenza virus type 3, are known to have evolved strategies that specifically inhibit GzmB \(^{41,42}\), and poxvirus can block caspase signaling \(^{11,43}\). Interestingly, in our study we found little to no caspase-3 activation in bronchial epithelial cells, clearly the most important site for PVM replication \(^{27}\). However it is currently unknown whether PVM directly induces anti-apoptotic pathways in infected bronchial epithelial cells.

Similar to previous findings \(^{9}\), PVM infection was associated with increased caspase-3 activation in alveolar wall cells, and this was markedly reduced in Gzm-deficient mice. As such, this finding may explain at least in part the observed decreased lung permeability in the Gzm-deficient mice. Accumulating evidence implicates caspase-dependent apoptosis as an important mechanism leading to loss of alveolar capillary barrier integrity and ultimately enhanced lung permeability \(^{44}\). Patients who die with ALI/ARDS have increased caspase-3 staining in alveolar wall cells \(^{45}\), and numerous experimental studies modelling both indirect and direct ALI/ARDS have shown enhanced apoptosis of lung epithelial cells is associated with leakage of serum proteins into the lungs and histopathological alterations, which can be attenuated by inhibiting pro-apoptotic pathways \(^{44;46;47}\). Our findings suggest the Gzm pro-apoptotic pathway can contribute to the development of alveolar injury in severe pneumovirus disease. Interestingly, both GzmA and GzmB mRNA expression is augmented in patients with septic ARDS and in mice intratracheally instilled with LPS \(^{47,48}\), suggesting the Gzm pathway may also be of relevance to bacterial-induced ALI/ARDS.

In addition to pro-apoptotic functions of Gzms, recent renewed attention has been given to their potential pro-inflammatory effects, specifically of GzmA \(^{18,19}\). Metkar et al. \(^{18}\) found that active human GzmA induces the release of IL-1\(\beta\), IL-6 and TNF-\(\alpha\) from monocytes, which can be attenuated by the IL-1\(\beta\) convertase (caspase-1) inhibitor WEHD-FMK, and similarly, that mouse macrophages produce IL-1\(\beta\) in response to treatment with active mouse GzmA. Interestingly, GzmA and GzmB-cluster deficient mice showed a delayed lung neutrophil response to PVM infection, but this was not associated with the release of IL-1\(\beta\), IL-6, TNF-\(\alpha\), KC and MIP-2. The fact that we did not detect IL-1\(\beta\) in the BALF of any of the mice may suggest that the above mentioned mechanism of GzmA-mediated pro-inflammatory activity through caspase-1 may not be directly relevant in the PVM natural infection model. IFN\(\gamma\) has been identified as a critical regulator of neutrophil influx into the lungs in severe PVM disease in mice \(^{32}\), and followed the same pattern as the total neutrophil counts in the GzmA and GzmB-cluster deficient mice. However, it remains to be elucidated whether mouse Gzm may directly mediate the release of IFN\(\gamma\), as for example, human GzmA does not induce IFN\(\gamma\) specifically in monocytes \(^{18}\). In addition, one needs to recognize that a number of different events, including adhesion, migration and clearance, affect the number of neutrophils in the lungs. In this light, the potent proteolytic degradation of extracellular matrix proteins by both GzmA and GzmB is of particular interest, because Gzm-mediated remodelling of the extracellular
environment has been shown to have important consequences for cellular migration and survival. As stated above, in the most current paradigm, GzmA elicits predominantly pro-inflammatory activity, while GzmB mediates caspase-dependent apoptosis, based mostly on observations in vitro. However, deciphering the specific functions of the individual Gzm family members in vivo has proven to be difficult and not straightforward. Interestingly, studies in mice have shown that the combined deficiency for GzmA and the GzmB-cluster can result in a more pronounced or paradoxically diminished phenotype, as compared to single-gene (GzmA or the GzmB-cluster) deficiency, suggesting complex and synergistic interaction between Gzms. The interpretation of experiments involving Gzm targeted mice is further complicated by the fact that the GzmB-gene deleted mice lack the entire GzmB-cluster which includes GzmC, -D and -F. In addition, differences in substrain background between wild type, single-gene and double-gene Gzm deleted mice may affect host responses to pathogens. Finally, in vivo, through indirect mechanisms involving inflammatory cells and extra-cellular matrix remodeling, Gzms may influence several processes, including cell death pathways, to which they are not directly attributed to: e.g. while GzmA does not directly cause caspase activation, in our study, GzmA single-gene deleted mice showed some reduction in lung caspase-3 activity. Taking into account these several issues, we have primarily focused on the effects of combined deficiency for GzmA and the GzmB-cluster, but this obviously limits our interpretation of the functions of the individual Gzm family members in acute pneumovirus infection.

Finally, it is important to recognize that mouse and human Gzm differ in structure, enzyme activity and inhibitory regulation. For example, human GzmB is more cytotoxic than mouse GzmB in vitro. In addition, unlike mouse GzmB, the human form requires Bid cleavage for full caspase-3 activation, and is controlled by the inhibitor PI-9. Furthermore, the evolution of the Gzm system, potentially driven by host specific pathogens, may compromise some of the meaning of any study of Gzm responses to human virus pathogens inoculated in mice. As such, the use of the natural mouse pneumovirus pathogen, PVM, in this work may represent an important advantage, as it permits us to explore the roles and functions of Gzms in response to a physiologically and evolutionarily relevant challenge. On the other hand, although PVM and RSV are both pneumoviruses, they are not the same virus, and we need to stress there is no one animal model that displays all features of human RSV disease.

In conclusion, pneumovirus infection in mice induces expression of GzmA and GzmB by effector lymphocytes. The combined deficiency of GzmA and the GzmB-cluster results in a delayed progression of pneumovirus disease by reducing alveolar injury in mice, without altering viral clearance. We speculate that targeting of the Gzm system in humans may be beneficial in diminishing RSV-ALI/ARDS immunopathogenesis.
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Mechanical Ventilation Enhances Lung Inflammation and Caspase Activity in a Model of Mouse Pneumovirus Infection

Reinout A. Bem
Job B.M. van Woensel
Albert P. Bos
Amy Koski
Alex W. Farnand
Joseph B. Domachowske
Helene F. Rosenberg
Thomas R. Martin
Gustavo Matute-Bello

1 Pediatric Intensive Care Unit, Emma Children’s Hospital AMC, Amsterdam, The Netherlands; 2 VA Puget Sound Health Care System and 3 Center for Lung Biology, Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of Washington School of Medicine, Seattle (WA); 4 SUNY Upstate Medical University, Syracuse (NY); 5 Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda (MD)

ABSTRACT

Severe infection with respiratory syncytial virus (RSV) in children can progress to respiratory distress and acute lung injury (ALI). Accumulating evidence suggests that mechanical ventilation (MV) is an important cofactor in the development of ALI by modulating the host immune responses to bacteria. This study investigates whether MV enhances the host response to pneumonia virus of mice (PVM), a mouse pneumovirus that has been used as a model for RSV infection in humans. BALB/c mice were inoculated intranasally with diluted clarified lung homogenates from mice infected with PVM strain J3666 or uninfected controls. Four days after inoculation the mice were subjected to 4 hr of MV (V1 10 ml/kg), or allowed to breathe spontaneously. As compared with mice inoculated with PVM-only, the administration of MV to PVM-infected mice resulted in increased bronchoalveolar lavage fluid (BALF) concentrations of the cytokines MIP-2, MIP-1α (CCL3) and IL-6; increased alveolar-capillary permeability to high molecular weight proteins; and increased caspase-3 activity in lung homogenates. We conclude that MV enhances the activation of inflammatory and caspase cell death pathways in response to pneumovirus infection. We speculate that MV potentially contributes to the development of lung injury in patients with RSV infection.
**INTRODUCTION**

Respiratory syncytial virus (RSV) is an important cause of severe lower respiratory tract infections in infants and young children \(^1\). Treatment remains largely limited to supportive measures such as mechanical ventilation \(^2\). Severe RSV infection may progress to acute lung injury (ALI) or its more severe form, the acute respiratory distress syndrome (ARDS) \(^3;4\). ALI/ARDS is a syndrome associated with prominent neutrophil recruitment to lung tissues accompanied by increased permeability of the alveolar epithelial barrier. The development of ALI/ARDS in children with RSV infection frequently necessitates prolonged mechanical ventilation, which may lead to long-term morbidity and related functional consequences \(^4;6\). At the present time, the factors that determine whether RSV infection progresses to ALI/ARDS remain unclear.

Recent studies suggest that mechanical ventilation may contribute to the development of lung injury by enhancing the host response to bacteria and bacterial products \(^7;11\). Mechanical ventilation with tidal volumes of 10 ml/kg amplifies the pulmonary inflammatory responses of mice to transpharyngeal LPS by a mechanism involving activation of genes involved in inflammatory and stress responses, including the genes coding for the cytokines CCL3 (MIP-1\(\alpha\)), CXCL2 (MIP-2) and IL-6; components of the mitogen-activated protein kinases (MAPK) pathway (e.g. GADD45-\(\alpha\), \(\beta\) and \(\gamma\)), and transcription factors (e.g. IRF-7) \(^8\). O’Mahony *et al.* extended these findings by demonstrating that mechanical ventilation enhances the lung inflammatory response to systemic LPS, and Dhanireddy *et al.* confirmed that mechanical ventilation enhances lung injury in response to live bacteria \(^10;11\). The exact mechanisms whereby mechanical ventilation alters the host inflammatory response remain unclear, but studies *in vitro* have shown that the application of cyclic pressure-stretch to primary human alveolar macrophages and alveolar epithelial-like A549 cells induces IL-8 release, which is enhanced when the cells are exposed to LPS \(^12;14\). These findings suggest that mechanical ventilation results in an inflammatory response that may be augmented by co-stimulation with bacteria. One important question is whether the interaction between mechanical ventilation and host defences is exclusive to bacteria or bacterial products, or instead is a more general phenomenon extensive to viral pathogens such as RSV.

A limitation to the study of pathogenic mechanisms of RSV infection *in vivo* is the relative resistance of mice to RSV \(^15;16\). RSV shows limited replication in mouse lungs, and causes little to no overt clinical disease in mice even when inoculated with high titers \(^16\). As an alternative, a mouse model using pneumonia virus of mice (PVM) is being increasingly recognized as having unique features that mimic human RSV lower respiratory tract infections \(^15;17\). PVM is a natural rodent pathogen belonging to the same virus family, subfamily and genus as RSV, and shows robust replication in mice \(^15;17\). PVM and RSV share the same gene order and are structurally similar to one another \(^18;19\). Most importantly, murine PVM and human RSV infections induce similar clinical and
pathological responses, including predominance of peribronchiolar cellular inflammation in the lungs and a cytokine profile dominated by CC and CXC chemokines \(^{15,20-23}\). It is important to emphasize that RSV and PVM are different viruses, and therefore PVM remains an imperfect model of human RSV. However, because of the lack of better murine models of RSV infection, and because the responses elicited by PVM in mice are similar to those elicited by RSV in humans, we used PVM in this study as a model to investigate acute lung injury and inflammation by mechanical ventilation.

In this study, we test the hypothesis that mechanical ventilation enhances the lung response to lower respiratory tract infection by PVM in mice by investigating the lung inflammatory, permeability and apoptotic responses of mice subjected to mechanical ventilation 4 days after intranasal inoculation with the virulent PVM strain J3666.

**METHODS**

**Viral stock preparation**

Pneumonia virus of mice (PVM) strain J3666, was a gift from Dr. A.J. Easton and was kept virulent by continuous passage in mice \(^{24}\). For this study, lungs from five BALB/c mice infected with PVM were homogenized at 4°C in 1 mL Iscove’s modified Dulbecco’s medium (IMDM) (Life Technologies, Gaithersburg, MD), then spun at 13,000 \( x \) \( g \) for 5 min at room temperature. The supernatants were pooled and stored as individual aliquots in liquid nitrogen. The virus titer in the aliquots was \( 1.1 \times 10^{10} \) copies of PVM / ml. For control inoculations, clarified lung homogenates were generated from a healthy BALB/c mouse by identical methods. On the day of each experiment, one aliquot was thawed and diluted 30-fold in RPMI medium (Cellgro, Mediatech Inc, Herndon, VA).

**Animal protocols**

The animal protocols were approved by the Animal Care Committee of the VA Puget Sound Healthcare System, Seattle, WA. Female BALB/c mice weighing 18 to 22 g (Jackson Laboratory, Bar Harbor, ME) received intranasal instillations of diluted virus stock in a total volume of 80 µL. Four days later the mice were anesthetized with inhaled isoflurane. Some mice were allowed to recover from anesthesia, and some were intubated endotracheally and subjected to 4 hr of mechanical ventilation using tidal volume=10 ml/kg; respiratory rate=150 breaths/minute; fraction of inspired oxygen=0.21; and PEEP= 2 cm H\(_2\)O \(^{11}\). Airway pressures and electrocardiogram were monitored continuously, and the rectal temperature was kept at 36-37°C with external heating. At the end of the experiment the mice were euthanized with intraperitoneal pentobarbital (120 mg/kg) and exsanguinated by closed intracardiac puncture. The left lung was removed and flash-frozen in liquid nitrogen. Bronchoalveolar lavage (BAL) was performed in the right lung by instilling four separate 0.5 ml aliquots of 0.9 % NaCl containing 0.6 mM EDTA. An aliquot of BAL fluid (BALF) was processed immediately
for cell counts and differentials. The remainder of the BALF was spun at 200 x g for 10 min at 4°C and the supernatants were stored in individual aliquots at -80°C. Immediately after the BAL procedure, the right lung was fixed with 4% paraformaldehyde at 15 cm H₂O for histological analysis.

**Experimental design**

First, to determine the optimal day for mechanical ventilation, defined as the onset of inflammation, mice received intranasal instillations of clarified lung homogenates containing PVM, diluted 1:100, 1:30 and 1:10 in RPMI medium; control mice were instilled with RPMI medium containing no virus (n=5/group). The mice were evaluated for clinical evidence of disease once per day using a score previously tested in other PVM studies (Table 1) (modified from Cook et al. 25). Next, to determine the optimal dose of virus, mice were euthanized at the onset of clinical signs of disease and evaluated for cellular, inflammatory, permeability and apoptotic responses.

Once the time and dose of virus were identified, we studied 4 groups of mice: mice inoculated with control homogenates followed by spontaneous breathing on day 4 (“SB”, n = 6); mice inoculated with control homogenates followed or mechanical ventilation on day 4 (“MV”, n = 5); mice inoculated with PVM followed by spontaneous breathing on day 4 (“PVM + SB”, n = 6) and mice inoculated with PVM followed by mechanical ventilation on day 4 (“PVM + MV”, n = 6).

**Measurements**

**Lung Injury.** Total cell counts were performed on an aliquot of the BALF, using a hemacytometer. Differential cell counts were performed on cytospin preparations using the Diff-quick method (Andwin Scientific, Addison IL). BALF total protein was measured with the bicinchoninic acid method (BCA assay, Pierce Co., Rockford, IL). BALF IgM, α-macroglobulin, and MIP-1α (CCL3) were measured with immunoassays (Bethyl Laboratories Inc., Montgomery, TX for Ig-M; Life Diagnostics, Inc. West Chester, PA for α-macroglobulin and R&D systems, Minneapolis, MN for MIP-1α). BALF TNF-α, IL-1β, MIP-2, KC, IL-6, IL-10, MCP-1 (CCL2), IFN-γ, GM-CSF, and VEGF were measured using a multiplex fluorescent bead assay (R&D systems, Minneapolis, MN).

**Table 1.** Scores associated with clinical signs caused by PVM infection in mice.

<table>
<thead>
<tr>
<th>Clinical signs</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy, no signs of illness</td>
<td>1</td>
</tr>
<tr>
<td>Subtle ruffled fur</td>
<td>2</td>
</tr>
<tr>
<td>Evident ruffled fur with hunched posture</td>
<td>3</td>
</tr>
<tr>
<td>Evident lethargy with abnormal breathing pattern</td>
<td>4</td>
</tr>
<tr>
<td>Moribund</td>
<td>5</td>
</tr>
<tr>
<td>Dead</td>
<td>6</td>
</tr>
</tbody>
</table>
Apoptosis. Caspase-3 activity was measured in lung homogenates with the caspase-3/CPP32 Fluorometric Assay kit according to manufacturer instructions (Biovision Inc.). The concentrations of FasL and granzyme B in lung homogenates were measured using immunoassays (R&D systems, Minneapolis, MN and eBioscience, San Diego, CA respectively). Caspase-3 immunohistochemistry was performed using rabbit anti-cleaved caspase-3 mAb (BD Pharmingen, San Jose, CA) and biotinylated goat anti-rabbit IgG (Vector Laboratories) as previously described previously 26. DNA nick-end labeling assays (TUNEL) were performed using the TACS XL Blue Labeling kit from Trevigen Inc. (Gaithersburg, MD). TUNEL results were quantified by counting the number of positive cells in 12 randomly generated high power fields. The counts were done in a blinded fashion.

Viral loads. The viral sh gene (GenBank: AY573815) was used as marker of PVM. RNA was isolated from frozen lungs with the Absolutely RNA® Miniprep kit (Stratagene, La Jolla, CA) and reverse transcribed to cDNA (High-capacity cDNA kit, Applied Biosystems, Foster City, CA). Copies of the sh gene of PVM were detected in RT-PCR reactions containing 1 µl cDNA, Taqman PCR Master Mix (Applied Biosystems, Foster City, CA), 77 nM TAMRA probe (5’-6FAM-CGCTGATATGGCCTGACGA TAMRA-3’), 200 nM primers (5’-GCCTGATATGGCCTGACGA TAMRA-3’ and 5’-GCCTGATATGGCCTGACGA TAMRA-3’) (Ellis et al. 2007). The gapdh housekeeping gene was detected in cDNA samples using rodent gapdh primers (100nM) and VIC-probe (200nM) of (Applied Biosystems). Standard curves with known concentrations of the full-length sh gene and gapdh decatemplate (Ambion, Foster City, CA) were used for quantification. Results are expressed as copies of PVM-sh per 10^9 copies of gapdh 27.

Statistical analysis
Comparisons between multiple groups were performed using one-way ANOVA. Significance between groups was determined with the Fisher’s Least Significant Difference (LSD) post-hoc test. A p value of < 0.05 was considered statistically significant. Data are reported in the text as means ± SEM, and shown in the figures as individual data points and boxplots depicting the median, interquartile range and range.

RESULTS

Determination of time for mechanical ventilation and dose of PVM
The mice instilled with 1 x10^7 copies of PVM, and mice instilled with medium only developed no clinical evidence of disease (Figure 1A). The mice instilled with 3 x10^7 copies of PVM developed clinical evidence of disease on day 4 (mean clinical score = 3), and the mice treated with 9 x10^7 copies of PVM developed a clinical score of 2 on day 3 and a clinical
score of 5 on day 4. Based on these data, we chose day 4 as the day for the ventilation studies. Next, to confirm the dose of virus, we evaluated the key variables of lung injury on day 4. Mice treated with $3 \times 10^7$ copies of PVM developed mild increases in total BALF PMN and cytokines (Figure 1B and C), and minimal increases in permeability and apoptosis markers (Figure 1D and E). Based on these data, we chose the dose of $3 \times 10^7$ copies of PVM as the optimal dose to identify an enhancing effect of mechanical ventilation.

**Figure 1.** Mean clinical score in mice treated with medium only (no virus) or 3 different doses of PVM (low: $1 \times 10^7$; mid: $3 \times 10^7$; high: $9 \times 10^7$ copies of PVM), evaluated on days 1 – 4 after inoculation (A). Total BALF macrophages (AM), neutrophils (PMN) and lymphocytes (Ly) (B); BALF cytokine concentrations (C); BALF permeability markers (IgM and $\alpha$-macroglobulin) (D) and lung homogenate caspase-3 activity (E) in mice treated with medium only (no virus) or 3 different doses of PVM (low: $1 \times 10^7$; mid: $3 \times 10^7$; high: $9 \times 10^7$ copies of PVM) on day 4 after inoculation. n= 5 per group. * p < 0.05 as compared to the uninfected mice.
Lung leukocyte response

The number of alveolar macrophages in the BALF of uninfected, spontaneously breathing mice was $41.5 \pm 5.0 \times 10^4$ cells (Figure 3A). In comparison, the number of macrophages in the BALF of each of the other groups was significantly decreased: $26.1 \pm 2.9 \times 10^4$ cells in mice exposed to mechanical ventilation alone; $21.3 \pm 4.7 \times 10^4$ cells in PVM-treated mice breathing spontaneously, and $18.0 \pm 2.4 \times 10^4$ cells in mice exposed to both PVM and mechanical ventilation. In contrast, significant neutrophil recruitment was observed in response to PVM infection ($1.6 \pm 0.9 \times 10^4$ cells), but mechanical ventilation did not result in a large additional response ($2.3 \pm 0.9 \times 10^4$ cells) (Figure 3B). PVM infection resulted in lymphocyte recruitment, and the addition of mechanical ventilation augmented lymphocyte recruitment approximately 4-fold (Figure 3C).

Lung cytokine response

PVM infection is accompanied by local production of numerous cytokines, including CC and CXC chemokines. In particular, MIP-2 and MIP-1α (CCL3) correlate with the severity of murine PVM infection and of human RSV disease, and MIP-1α (CCL3) is a key mediator for granulocyte recruitment to lung tissue following PVM infection. In the present study, the concentrations of the chemokines MIP-2 and MIP-1α (CCL3) showed a small increase in spontaneously breathing mice infected with PVM, and a greater increase in PVM infected mice subjected to mechanical ventilation (Figure 4A-B). The concentrations of KC and IL-6 in were also significantly increased in the PVM + MV mice (Figure 4C-D). The concentrations of VEGF showed a trend towards increase in response to mechanical ventilation plus PVM.

Lung viral loads

The virus titer in the lungs of mice infected with PVM and allowed to breathe spontaneously (PVM + SB) was $1.3 \pm 0.6 \times 10^8$ copies per $10^9$ copies GAPDH; this was similar to that of mice infected with PVM and subjected to mechanical ventilation (PVM + MV) ($0.7 \pm 0.4 \times 10^8$ copies per $10^9$ copies GAPDH, Figure 2). No viral RNA was detected in the uninfected mice inoculated with diluted control homogenates.
Figure 3. Total BALF macrophages (AM, A), neutrophils (PMN, B) and lymphocytes (C) detected in uninfected mice allowed to breathe spontaneously (SB, n = 6) or subjected to mechanical ventilation (MV, n = 5); and in PVM infected mice allowed to breathe spontaneously (PVM + SB, n = 6) or subjected to mechanical ventilation (PVM + MV, n = 6). * p < 0.05. Data are shown as individual data points and boxplots depicting the median, interquartile range and range.

Infection (Figure 4E). The cytokines TNF-α, IL-1β, IL-10 and GM-CSF were all below the limit of the assay. Likewise, low levels of MCP-1 (CCL2) and IFN-γ were detected in the BALF of mice of PVM infected mice only (data not shown).

Lung permeability
Lung permeability was assessed by measuring the BALF concentrations of total protein and of the high-molecular weight serum proteins IgM and α-macroglobulin. In uninfected mice breathing spontaneously the mean IgM concentration in the BALF was 33.7 ± 8.9 ng/ml (Figure 5A). Mechanical ventilation alone or PVM alone resulted in a small increase of IgM in BALF (69.4 ± 10.9 ng/ml for the MV group and 43.0 ± 10.2 ng/ml for the PVM + SB group); these increases did not reach statistical significance. In contrast, the mean IgM concentration in the BALF of mice exposed to both PVM and mechanical ventilation was 101.7 ± 20.3 ng/ml; this represented a significant increase compared to spontaneously breathing mice with or without PVM infection. The α-macroglobulin concentrations in BALF followed a similar pattern to IgM (Figure 5B), and the total protein in BALF showed a trend towards increased concentrations in the mice exposed to both PVM and mechanical ventilation (Figure 5C).
Lung apoptotic response

To assess the apoptotic response we measured lung homogenate caspase-3 activity, tissue expression of cleaved caspase-3 by immunohistochemistry, TUNEL staining, and lung homogenate concentrations of sFasL and Granzyme-B. Mechanical ventilation did not alter caspase-3 activity in the uninfected mice, but led to a significant increase in caspase-3 activity in the mice infected with PVM (Figure 6A). Caspase-3 activity was localized to cells of the
alveolar walls and macrophages, and this was independent of the treatment group (Figure 6E-H). Overall, there was less than 1 cell staining for caspase-3 in each high power field. TUNEL staining was increased in all of the mice infected with PVM, regardless of mechanical ventilation (p=NS) (Figure 6B). There was a trend towards increased concentrations of sFasL and granzyme B in the lungs of the PVM infected mice subjected to mechanical ventilation, but this did not reach statistical significance (Figure 6C, D).

**Microscopic lung histology**

There were no prominent differences observed in an examination of lung tissue from uninfected mice subjected to mechanical ventilation compared to those breathing spontaneously (Figure 7A and 7B). In the mice from the PVM + SB group, the lungs showed variable degrees of mixed mononuclear and neutrophilic peribronchial infiltrates (Figure 7C), consistent with previous findings. The lungs of the PVM infected mice subjected to mechanical ventilation showed tissue findings similar to those of the PVM + SB mice (Figure 7D).

**Figure 5.** Concentrations of IgM (A), α-macroglobulin (B) and total protein (C) in the BALF of uninfected mice allowed to breathe spontaneously (SB, n = 6) or subjected to mechanical ventilation (MV, n = 5); and in PVM infected mice allowed to breathe spontaneously (PVM + SB, n = 6) or subjected to mechanical ventilation (PVM + MV, n = 6). * p < 0.05. Data are shown as individual data points and boxplots depicting the median, interquartile range and range.
Figure 6. Caspase-3 activity in lung homogenates (A); number of TUNEL-positive cells per 12 high-power fields in lung tissue sections (B); BALF soluble FasL concentrations (C) and BALF granzyme B (GrB) concentrations (D) in uninfected mice allowed to breathe spontaneously (SB, n = 6) or subjected to mechanical ventilation (MV, n = 5); and in PVM infected mice allowed to breathe spontaneously (PVM + SB, n = 6) or subjected to mechanical ventilation (PVM + MV, n = 6). * p < 0.05. The cellular distribution of caspase-3 activation is shown in merged differential interference contrast (DIC) and fluorescence images (cleaved caspase-3 in pink, nuclei in blue) from a mouse in the SB group (E); MV group (F), PVM group (G), and PVM + MV group (H). The figures show the presence of signal in cells of the alveolar walls (open arrows) or macrophages (closed arrow).
Functional lung responses in ventilated mice

To assess whether the patchy distribution of peribronchial inflammation led to increases in airway flow resistance, we measured peak airway pressures continuously for the duration of mechanical ventilation. The peak inspiratory pressures in the MV and PVM + MV mice were similar during the period of mechanical ventilation (Figure 7E). In both mechanically ventilated groups, the peak inspiratory pressures increased with time. The transcutaneous CO₂ pressure in the mice in the MV group was 46 ± 2.2 mmHg, and 54 ± 2.8 mmHg in the PVM + MV group (p = 0.09).

Figure 7. H&E stained lung tissue sections of lung tissue from uninfected mice allowed to breathe spontaneously (SB, A) or subjected to mechanical ventilation (MV, B); and in PVM infected mice allowed to breathe spontaneously (PVM + SB, C) or subjected to mechanical ventilation (PVM + MV, D); 400X magnification. Note the peribronchiolar cellular infiltration in both the PVM + SB and PVM + MV group (arrows). E, mean peak inspiratory pressures in uninfected mice subjected to mechanical ventilation (n = 5) and PVM infected mice subjected to mechanical ventilation (n = 6). Means ± standard error.
DISCUSSION

The goal of this study was to determine whether mechanical ventilation enhances the response of mice to pneumoviral infection. The mice were infected with PVM and subjected to a mechanical ventilation strategy that results in minimal injury when applied alone. The main findings are that exposure to 4 hours of mechanical ventilation in the setting of PVM infection enhances the local release of the cytokines MIP-2, MIP-1α (CCL3) and IL-6; increases alveolar-capillary permeability to high molecular weight proteins; and induces an increase in lung caspase-3 activity. These data suggest that mechanical ventilation may serve to exacerbate local inflammatory responses and lung injury in pneumovirus infection.

Human and animal studies have demonstrated that mechanical ventilation may worsen lung injury and outcome in children and adults with ALI/ARDS. In adults, the ARDS Network trial showed that ALI/ARDS mortality can be decreased by reducing tidal volumes. Interestingly, in the same cohort lower tidal volumes were also associated with lower circulating concentrations of pro-inflammatory cytokines such as IL-8 and IL-6, suggesting the existence of a direct link between mechanical ventilation and inflammation. In the pediatric population a recent retrospective study evaluating children with ALI/ARDS showed that the change in ventilatory practice towards the use of a lower tidal volume in 2000-2004 was associated with decreased mortality and increased ventilator-free days. Thus, tidal volumes are associated with mortality and with the severity of the inflammatory response in adults and children with ALI/ARDS. In contrast to patients with ALI/ARDS, patients with spinal cord injury or other neuromuscular diseases that need mechanical ventilation usually receive large tidal volumes, and yet these patients do not develop lung injury. Thus, the association between tidal volume, mortality and inflammation seen in patients with ALI/ARDS is not dependent exclusively on mechanical factors associated with stretch, but requires the presence of additional factors associated with the ALI/ARDS lung microenvironment, that are magnified by the addition of mechanical ventilation.

The possibility that mechanical ventilation enhances the inflammatory response of the lungs to pathogens has been underscored by studies demonstrating that mechanical ventilation at tidal volumes that do not induce injury in healthy lungs, may induce injury in lungs exposed directly or indirectly to bacterial products or to live bacteria. The synergism between mechanical ventilation and bacteria/bacterial products is characterized by increased alveolar neutrophils, increased cytokine concentrations, and disruption of the epithelial barrier. The present study demonstrates that in addition to enhancing the lung response to LPS and bacteria, mechanical ventilation also enhances the lung response to viruses such as PVM. The amplifying effect of mechanical ventilation on PVM infection has several features in common with previous models based on LPS or bacteria, including increased release of MIP-2, MIP-1α (CCL3) and IL-6. This suggests that the enhancing effect of mechanical ventilation on lung injury is not
limited to bacteria or LPS, but instead represents a more general response to a variety of viral and bacterial pathogens that signal through different receptor pathways. This has broad implications for the treatment of critically ill patients.

The cellular mechanisms linking mechanical ventilation with enhancement of the innate immune response to pathogens remain unclear. Human macrophages and alveolar epithelial cells exposed to cyclic stretch in vitro show alterations in membrane lipid trafficking and changes in cytoskeleton-protein interactions that result in activation of mitogen-activated protein kinases (MAPK) including JNK and p38, and activation of transcription factors such as NF-κB, Erg-1 and the AP-1 component c-fos. Activation of pro-inflammatory pathways following these intracellular processes, most notably the activation and translocation of NF-κB, is thought to be a key event in stretch induced lung injury in vivo. The magnitude of the cytokine response of lung cells to in vitro stretch varies with the cell type, with macrophages showing greater release of TNF-α, IL-8 and IL-6 as compared to epithelial cells. In addition to the induction of pro-inflammatory events, stretch induced by mechanical ventilation may result in the activation of cell death pathways. Mice exposed to 4 hr of mechanical ventilation with tidal volumes of 20 ml/kg show evidence of caspase-dependent apoptosis in lung endothelial and alveolar epithelial cells by a mechanism involving activation of the MAPK member p38. Together these data suggest that stretch activates intracellular pathways leading to inflammatory and cell death responses in epithelial and immune cells of the lungs.

The inflammatory and cell death responses of lung cells to stretch are modulated by bacterial products. The release of pro-inflammatory cytokines by human macrophages and human and rat lung epithelial cells exposed to stretch is enhanced by LPS in vitro. Tremblay et al. showed increased concentrations of TNF-α, MIP-2 and IL-6 in BALF of ex vivo rat lungs exposed to LPS, as compared to saline, after high volume ventilation. These observations have been extended by studies showing that unique sets of genes are upregulated in mice exposed to the combination of LPS and mechanical ventilation. These genes include the chemokines CCL3 and CXCL2; IL-6, IL-1β and components of the MAPK cascade such as GADD45-γ. A transcriptional analysis performed on the same dataset revealed enrichment of several transcription factors including NF-κB, ETF, USF2, IRF7 and Myc. These findings indicate that the interaction between mechanical ventilation and pathogens results in the activation of a specific transcriptional response that may result in enhancement of downstream pro-inflammatory pathways.

A number of studies suggest that apoptosis of lung epithelial cells and migrating leukocytes, such as neutrophils and macrophages, is a key event in the development of ALI/ARDS in adults. As mentioned above, the same may be true in children with severe RSV, who have marked caspase-3 activation in the airway epithelium; and studies in vitro showed that alveolar epithelial cells isolated from septic rats are more susceptible to cell death in response to cyclic stretch than alveolar epithelial cells from non-septic
rats. This suggests that stretch due to mechanical ventilation may enhance cell death pathways in the lungs. In our study, mice infected with PVM and subjected to mechanical ventilation had increased caspase-3 activity, which was primarily localized to cells in the alveolar walls and to macrophages; however, the number of TUNEL-positive cells was not significantly increased by mechanical ventilation. One possible explanation for the discrepancy between the observed caspase-3 activity and TUNEL staining is that caspase-3 activation is generally considered an early event in apoptosis, whereas DNA fragmentation occurs at later stages. Alternatively, the activation of caspases may trigger pathways separate from apoptosis, in particular pathways leading to inflammation. Therefore, the role of apoptosis in the synergism between mechanical ventilation and viral pathogens remains to be fully clarified.

The presence of caspase activation in cells of the alveolar walls and also in alveolar macrophages raises the question of whether the mechanism of caspase activation was the same for these two cell types. Some bacterial agents, such as *Legionella pneumophila* and *Streptococcus pneumoniae*, are capable of inducing apoptosis of both alveolar epithelial cells and macrophages; in contrast other agents, such as *Chlamydia muridarum* induce apoptosis in macrophages only. RSV has been associated with both enhancement and inhibition of apoptosis of immortalized lung epithelial cell lines *in vitro*. This differential effect of RSV on apoptosis may be explained in part by predominant effects of different RSV proteins such as the F protein and nonstructural proteins NS1 and NS2. SH induces p53-dependent apoptosis in A549 cells by a mechanism involving activation of the caspase-pathway, eventually resulting in cell shedding, in contrast NS1 and NS2 delay apoptosis by a pathway that may involve NF-κB. RSV infected A549 epithelial cells seem more susceptible for death receptor ligands such as FasL and TRAIL. Finally, studies of lung tissue of children with fatal RSV infection showed marked caspase-3 staining of the bronchiolar epithelium, suggesting a pro-apoptotic effect of RSV infection *in vivo*. Thus, the effects of infectious agents on apoptosis of lung cells appear to depend on the species of the infectious agent and on what specific pathogen pathways are triggered, and may involve enhancement or inhibition of apoptosis in epithelial cells, macrophages or both. The present study suggests that both macrophage and epithelial cell apoptosis are present in mice infected with PVM, but additional studies will be necessary to determine whether the mechanism of caspase activation is similar in these two cell types. The present study has several limitations. First, PVM infection resulted in patchy areas of peribronchiolar inflammation, which may have caused heterogeneous distribution of the tidal volume with some lung areas becoming exposed to higher stretch than others. The peak airway pressures were similar in the uninfected and PVM infected ventilated mice, suggesting the absence of large differences in airway obstruction between the two groups. However, peak airway pressures may not entirely reflect alveolar pressures, and we cannot fully exclude enhancement effects related to regional overdistention. As observed in studies using LPS, in the present study the mice...
treated with both mechanical ventilation and PVM showed greater variability in several measurements of lung injury\textsuperscript{7,10}. This observation may suggest differential responses of lung cells to mechanical forces, potentially resulting from regional volume/pressure differences in diseased lungs. Second, all of the mice subjected to mechanical ventilation in this study were kept anaesthetised with inhaled isoflurane. Volatile anesthetics appear to have immuno-modulatory effects\textit{in vitro}, but these are associated with immune suppression rather than activation\textsuperscript{57}. However, we cannot fully exclude that some of our findings are due to immunomodulation by the anesthetic used in this study. Finally, the PVM mouse model remains an imperfect model of human RSV infection, because PVM and RSV are not the same virus. However, because RSV is not a natural pathogen of mice, and because the molecular and pathological responses of mice to PVM share more features with human RSV infections than the murine responses to RSV, the PVM model has been used extensively as an experimental model of RSV infection\textsuperscript{15,17}.

In summary, mechanical ventilation enhanced the lung responses to viral infection caused by the murine pneumovirus PVM in mice. We conclude that mechanical ventilation may contribute to the pathogenesis of lung injury in response to pneumovirus infection by enhancing activation of inflammatory and cytopathological pathways in the lungs.
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Summary and General Discussion
In this final chapter I present a short summary (Table 1) and general discussion of our findings in a broader context. For the specific interpretations, limitations and conclusions of the individual studies in this thesis I refer to the corresponding chapters.

**ACUTE LUNG INJURY**

Acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) is a disease entity that poses a major challenge in the care for patients admitted to the intensive care unit (ICU). ALI/ARDS is defined as a) acute onset; b) severe arterial hypoxemia (\(\text{PaO}_2/\text{FiO}_2\) ratio \(\leq 300\) mmHg for ALI and \(\text{PaO}_2/\text{FiO}_2\) ratio \(\leq 200\) mmHg for ARDS); c) bilateral infiltrates on chest radiograph; and d) no evidence of left atrial hypertension (American-European Consensus Conference criteria \(^1\)). It can develop due to a wide variety of causes such as pneumonia, sepsis, trauma, burns or multiple transfusion, in patients with underlying diseases, such as cancer or immune disorders, but also in previously healthy individuals \(^2\). The unifying features of ALI/ARDS are considered to be extensive pulmonary inflammation and diffuse alveolar damage. However, the latter histopathological finding may be present in only half of the patients \(^3\). Currently, no specific treatment for ALI/ARDS exists, and therefore more insight into its pathophysiology is urgently needed.

**Children**

One of the factors that may affect the pathophysiology of lung injury is age. Children appear to be relatively protected in terms of incidence and outcome of ALI/ARDS \(^4\text{-}^7\). Large, multicenter studies using the AECC criteria have reported mortality rates of 20-35% for pediatric ALI/ARDS \(^4\text{-}^5\text{,}^8\), as compared to 40-55% for adults \(^6\text{-}^9\). Erickson et al. even found a positive correlation between age and ALI/ARDS mortality within a pediatric ICU patient cohort \(^8\).

So far, we can only speculate on what determines this age-related effect on the risk and course of ALI/ARDS. Apart from differences in the physical properties between developing lungs of young children and fully mature lungs in adults, a number of other factors including age-related differential (immune) responses of both structural and myeloid cells in the lungs may play an important role herein. At the same time, there are specific causes of lung injury in young children such as severe infection by respiratory syncytial virus (RSV), and infant (I)RDS of preterm birth that show a number of features overlapping with ALI/ARDS \(^10\text{-}^{11}\), and as such, this adds to the heterogeneity of pediatric ALI/ARDS, making a comparison between children and adults not straightforward.

**Pathogenesis**

The studies in this thesis have attempted to extend our insight of ALI/ARDS pathogenesis by focusing on the role of pro-apoptotic pathways in lung epithelial injury and inflammation in children.
Lung epithelial cell apoptosis

The first evidence for alveolar epithelial injury in humans who died with ARDS was presented in the study of Bachofen and Weibel. Loss of alveolar capillary barrier integrity by epithelial injury is now considered a key event in ALI/ARDS pathogenesis, consistent with the ‘epithelial cell hypothesis’ as discussed in the introduction of this thesis. The appearance of studies showing apoptotic markers such as DNA fragmentation, caspase activation and expression of Bcl-2 family members in alveolar wall cells in lung tissues from humans who died with ALI/ARDS, resulted in a search for specific pro-apoptotic mediators and their regulatory mechanisms that may be implicated in the development of epithelial injury in ALI/ARDS.

Most research in this field has been performed in adult humans and animals, although there is evidence that lung epithelial cell apoptosis also may play an important role in pediatric ALI/ARDS. On the other hand, lung development and maturation in young children and infants may affect the (dys)regulation of apoptosis during ALI/ARDS. During normal (postnatal) lung development there is a tight balance between anti- and pro-apoptotic pathways, at least partly mediated by stretch, and this ensures correct airway branching and alveolar septal thinning. De Paepe et al. showed that disruption of this tight balance, for example by inducing local expression of FasL in the perinatal period, causes deleterious effects on lung development and decreases postnatal survival in mice. Although the precise long term outcome of ALI/ARDS in children that survive is unclear, both restrictive and obstructive functional alterations have been reported. Interestingly, there is some limited data from animal models for ALI/ARDS, including treatment with hyperoxia and a combination of LPS and mechanical ventilation, that suggests young age is relatively protecting against harmful pro-apoptotic events. In line with these results, we found a positive correlation between age and active caspase-3 immunostaining in lung epithelial cells in children with ARDS. One possible explanation for age-related difference in the ALI/ARDS pro-apoptotic response is that the increased regulation of anti- and pro-apoptotic pathways during lung development prevents extensive unscheduled apoptosis induced by injurious hits such as bacterial infection, mechanical ventilator-induced stretch and hyperoxia. In light of the lower ALI/ARDS incidence and mortality in young children, our study underscores the need for further insight into the regulation of apoptosis in pediatric ALI/ARDS.

Pro-apoptotic pathways

Soluble pro-apoptotic mediators that target lung epithelial cells, and are released in the lungs during ALI/ARDS are potential targets for therapeutic inhibitors. In an elegantly designed series of studies, Matute-Bello et al. have demonstrated this for soluble FasL (sFasL) belonging to the Fas/FasL pro-apoptotic pathway: they showed that biologically active sFasL is present in the epithelial lining fluid of humans with ALI/ARDS in concentrations high enough to induce apoptosis in lung epithelial cells in vitro, that Fas activation in the lungs of animals
results in lung injury, and finally, that by blocking Fas/FasL signaling lung injury in response to live bacteria or LPS is reduced\(^{24-28}\). Several other investigators have found similar results, confirming the potential role and clinical relevance of the Fas/FasL system in ALI/ARDS\(^{29-34}\).

Besides the Fas/FasL system there are several other pro-apoptotic mechanisms, including the TRAIL death receptor-, granzyme-, and mitochondrial-pathway, that potentially promote epithelial injury during ALI/ARDS (chapter 1). In chapter 4-6 we studied the role of the TRAIL and granzyme pathways in a specific and frequent cause of ALI/ARDS in children: acute pneumovirus (RSV) infection. First, we found that the concentrations of the effector molecules of these pathways, sTRAIL and granzyme A/B respectively, in epithelial lining fluid of children with severe RSV disease are increased, as compared to children without pulmonary disease. Importantly, both sTRAIL and granzyme A/B released in the lung extracellular environment were found to be biologically active, and thus are not inactivated by decoy receptors, proteolytic enzymes or protease inhibitors, such as \(\alpha\)-2-macroglobulin\(^{35}\). Second, to address the question whether these pro-apoptotic mediators may actively play a role in epithelial injury during RSV infection, we performed additional in vitro cell culture and in vivo mouse experiments. In these, bronchial epithelial cells of children were found susceptible to human recombinant and natural sTRAIL-induced cell death in vitro (chapter 4), and gene targeting of granzyme A and the granzyme B-cluster resulted in decreased caspase-mediated apoptosis in alveolar wall cells and reduced lung permeability in response to acute pneumovirus infection in vivo (chapter 6).

The above results suggest that pro-apoptotic mediators such as sTRAIL and granzymes are implicated in RSV-ALI/ARDS pathophysiology, however, it remains unclear whether these findings may or may not be extrapolated to other etiologies of ALI/ARDS in both children and adults. It is very likely that RSV-induced ALI/ARDS may be very different from, for example, sepsis-induced ALI/ARDS, given the differences in disease course and mortality rates (in general RSV disease is not lethal upon supportive treatment)\(^{8;11}\). Our findings of decreased caspase-3 staining in lung tissues of young children who died with ARDS (chapter 2), while in the rare cases of fatal RSV marked caspase-3 staining has been reported\(^{36}\), further underlines the difficulties concerning the relatively high heterogeneity in ALI/ARDS with respect to underlying clinical disorders. In addition, virus-induced ALI/ARDS may differ from other etiologies of ALI/ARDS because anti-virus lymphocyte responses may be relatively prominent. For example, while potential sources of (s)FasL and (s)TRAIL in the lungs include a number of different myeloid and non-myeloid cells\(^{37-45}\), granzyme expression appears mainly confined to the lymphocyte compartment and thus may have greater impact in viral disease as compared to bacterial disease\(^{45;46}\) (chapter 5). On the other hand, augmentation of the granzyme pathway has been reported in human patients with septic-ARDS as well as a LPS animal model\(^{31;47}\). Whether different etiologies of ALI/ARDS provoke specific differential pro-apoptotic responses remains to be clarified further.
Interaction with inflammation

In the introduction of this thesis I described the ‘neutrophil hypothesis’ (mediated by inflammation) and the ‘epithelial cell hypothesis’ (mediated by apoptosis) in ALI/ARDS pathophysiology. However, rather than being separate entities the central events in these pathogenic mechanisms may be closely linked and interconnected. For example, activated leukocytes, including neutrophils, lymphocytes and monocytes/macrophages (chapter 4-6), are a potential source for (soluble) pro-apoptotic mediators that target lung epithelial cells (chapter 4-5). As such, initial pro-inflammatory activity in the lungs activates pro-apoptotic pathways leading to epithelial injury. Vice versa, initial activation of pro-apoptotic pathways may cause inflammation:

In the original paradigm, apoptosis is a relatively ‘silent’ event, providing a mechanism by which intact cells die without leakage of noxious cellular contents that may trigger undesirable inflammation. Indeed, programmed cell death during normal organ development and tissue remodeling occurs in the absence of an activated innate immune response. Rapid uptake of apoptotic cells by phagocytosis by macrophages or neighboring cells is essential in this process, and occurs by a complex series of events beginning with the recognition of specific apoptotic membrane alterations such as translocation of phosphatidylserine. Phagocytosis of apoptotic cells by macrophages inhibits the release of cytokines such as IL-1β, IL-8 and TNFα, providing a mechanism that limits tissue inflammation.

At the same time there is much evidence that challenges the view that apoptosis does not cause inflammation. First, cellular pathways that have traditionally been considered prototypical apoptotic may in fact have more diverse functions, including the activation of pro-inflammatory pathways. In chapter 3 we have presented in vitro evidence that activation of the Fas receptor in lung epithelial cells causes the release of the neutrophilic chemokine KC. Furthermore, we showed that intratracheal instillation of the Fas-activating antibody Jo2 causes lung injury associated with apoptosis as well as neutrophilic alveolitis and prominent cytokine responses in vivo, consistent with previous findings. Fas ligation activates the key pro-inflammatory transcription factor NF-κB in macrophages and lung epithelial cells, but how this interaction occurs remains unclear. In a current intriguing paradigm intracellular Fas-associated death domain (FADD) protein, which is necessary for the downstream caspase activation in Fas-mediated apoptosis, shuttles between the intracellular tail of Fas and the major LPS receptor Toll-like receptor (TLR)4 adaptor protein MyD88. FADD bound to MyD88 blocks TLR4 activation of NF-κB, but FADD bound to Fas after Fas ligation enables TLR4 pro-inflammatory activity, thereby providing a direct regulatory link between Fas-apoptosis and inflammation. Similar to the Fas/FasL system, the granzyme pathway also has the capacity to elicit pro-inflammatory activity, but the underlying mechanism is even less clear. For example, granzyme A induces the release of pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα from monocytes and epithelial cells, which is dependent on
caspase-1. Although we found a positive correlation between the local concentrations of extracellular granzymes and IL-8 and total cell counts in humans with severe RSV disease (chapter 5), there was no association between release of the abovementioned cytokines in the lungs and granzyme expression in pneumovirus-infected mice (chapter 6), suggesting this mechanism of granzyme-mediated inflammation may not be relevant for RSV disease.

Second, in disease states, such as ALI/ARDS, there are a number of indirect mechanisms that may trigger inflammation as a consequence of apoptosis. For example, the capacity of phagocytosis may simply be limited due to dysfunctional engulfment by macrophages or due to high numbers of apoptotic cells. Apoptotic cells that are not cleared undergo secondary necrosis resulting in cell membrane disruption and subsequent leukocyte activation. To date, most research in ALI/ARDS has focused on the rate of clearance of apoptotic neutrophils in the lungs. Several proteins, such as PAI-1 and HMGB-1, that are released into the lung microenvironment during inflammation affect macrophage-mediated neutrophil phagocytosis, however, there is limited knowledge with regard to the regulation of clearance of apoptotic lung epithelial cells. In addition, widespread apoptosis of parenchymal cells during disease exposes components of the extracellular matrix to migrating leukocytes. Areas of denuded alveolar basement membrane due to epithelial disruption are a prominent feature in histopathological studies of ALI/ARDS, and provide an important pro-inflammatory activation site.

Taken together, these findings suggest pro-apoptotic pathways may cause inflammation in the lungs by direct or indirect mechanisms, and this supports a close link between the ‘neutrophil hypothesis’ and the ‘epithelial cell hypothesis’ in ALI/ARDS pathogenesis.

CLINICAL IMPLICATIONS

Apoptosis-based therapies are currently exploited in a wide variety of diseases, including cancer and HIV/AIDS. However, before we can speculate on manipulating pro-apoptotic pathways to inhibit epithelial injury in ALI/ARDS in the ICU clinical setting, we need to recognize the complex and dynamic nature of apoptosis in the lungs. First, at a particular moment during disease the lungs may contain over thirty distinct cell types of non-myeloid and myeloid origin, and these all may have very different apoptotic responses during ALI/ARDS. For example, adding bronchoalveolar lavage fluid (BALF) of patients with ALI/ARDS to culture media delays neutrophil apoptosis, but induces lung epithelial cell death. This would infer that cell-specific pro-apoptotic mediators need to be targeted, leaving no place for broad-range apoptosis inhibitors such as caspase blockers. However, even within a group of cells, such as the lung epithelium, differential apoptotic responses to a single pro-apoptotic mediator exist depending on
the localization (e.g. distal versus proximal), and possibly the developmental stage/age of the lungs (chapter 7-2).

Second, we do not know the extent of cell death at which there is critical dysfunction of the lung epithelium in ALI/ARDS in vivo. Apoptosis is a dynamic process, and death receptor ligands and granzymes may induce cell death within hours, while under normal circumstances there may be swift repair of the lung epithelium. Studies in humans that provide evidence for lung epithelial apoptosis in ALI/ARDS are based on ‘snapshot’ (immuno)histochemical analyses of lung tissue specimens obtained at autopsy, and thus are very limited in the quantification and interpretation of apoptosis and outcome. This may in particular pose difficulties in the case of viral infection: elimination of virus-infected cells by apoptosis may be a highly beneficial anti-viral response, but at the point at which there is widespread viral infection, and thus excessive apoptosis, normal lung function may be compromised. Appreciating the different stages of ALI/ARDS, timing of pharmacological intervention is critical.

Third, as described above, inhibiting pro-apoptotic pathways in the lungs may not only affect apoptosis, but may also target local inflammation, either by a direct or indirect mechanism. Although this could be a powerful secondary effect, we need to realize that this may not always be beneficial to outcome, in particular in ALI/ARDS caused by infection. Despite the above issues, there are a number of animal studies that suggest pharmacological based treatment inhibiting pro-apoptotic signaling in ALI/ARDS can reduce disease and improve survival. Remarkably, even systemic treatment with the broad spectrum caspase inhibitor Z-VAD.fmk, which can affect apoptosis in a wide variety of cells in the body, prolongs the survival of mice with LPS-induced lung injury, and this is associated with less apoptosis in the lungs. Other, more specific local treatments, such as intratracheal instillation of a FasL decoy receptor or Fas-small interfering RNA resulted in reduction of both epithelial cell apoptosis and neutrophilic inflammation in the lungs of mice. Although these findings are promising, it is needless to say that testing the effectiveness of targeting pro-apoptotic pathways in humans with ALI/ARDS is hampered by the unavailability of an animal model that reproduces all the features of this human disease.

Beside pharmacological intervention, ‘simply’ altering ICU treatment protocols may also affect lung epithelial cell apoptosis in the lungs. There is much evidence that supportive measures such as mechanical ventilation and hyperoxia therapy, although indispensable to the patient, can promote lung injury in patients with ALI/ARDS. For example, the application of low tidal volume ventilation has been shown to significantly reduce mortality and inflammation in humans. In chapter 7 we provided evidence that mechanical ventilation may alter the activation of caspase-3, together with the release of cytokines, in the lungs of pneumovirus-infected mice. These data underline the importance of protective ventilator strategies, but it remains to be elucidated whether the application of lower stretch indeed effectively decreases lung epithelial cell apoptosis in ALI/ARDS in humans.
CONCLUSIONS

Accumulating evidence implicates epithelial injury by apoptosis in the pathogenesis of ALI/ARDS in both adult and pediatric patients. Pro-apoptotic mediators present in the lung microenvironment during ALI/ARDS are a potential target for therapeutic inhibition. This thesis has added to this research field by reaching the following conclusions:

1. there may be age-related differences in the ALI/ARDS pro-apoptotic response of lung epithelial cells (chapter 1-2).

2. activation of the Fas pro-apoptotic pathway causes lung injury resulting from apoptosis and neutrophilic inflammation, mediated in part by lung epithelial pro-inflammatory cytokine release (chapter 3).

3. pneumovirus-induced ALI/ARDS in children is associated with enhanced local release of sTRAIL, which induces lung epithelial cell injury by apoptosis in vitro (chapter 4).

4. pneumovirus-induced ALI/ARDS in children is associated with activation of the granzyme pathway (chapter 5).

5. targeting of the granzyme pathway delays the progression of acute pneumovirus disease, mediated in part by reducing apoptosis and lung permeability (chapter 6).

6. mechanical ventilation enhances the activation of inflammatory and apoptosis in response to pneumovirus infection (chapter 7).

DIRECTIONS FOR FUTURE STUDIES

Many questions related to the role of the ‘neutrophil hypothesis’ and ‘epithelial cell hypothesis’ and pro-apoptotic pathways in ALI/ARDS still remain to be answered. Specific recommendations for future research related to findings in the present thesis include:

1. further explore age-related differences in ALI/ARDS. Identify whether these are caused by an unique pattern of soluble pro-apoptotic and inflammatory mediators in the lung microenvironment versus epithelial cell intrinsic programs, by comparative studies between young children and adolescents/adults analyzing patient BALF and ex vivo responses of isolated lung epithelial cells.

2. further explore long-term outcome of ALI/ARDS. Identify potential long-term effects of ALI/ARDS in young children and infants, specifically focusing on lung development and function, by follow up studies.

3. further explore biological markers of ALI/ARDS disease severity. Identify soluble pro-apoptotic and inflammatory mediators that serve to predict ALI/ARDS development and outcome, in specific patient groups or in general, by analyzing patient plasma and BALF in prospective studies.
4 further explore treatment options in ALI/ARDS. Identify potential new therapeutic strategies by pharmacological inhibitors of pro-apoptotic pathways, including the TRAIL and granzyme system, in mouse models for ALI/ARDS.

**Table 1. Summary of the studies**

<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>Species</th>
<th>Description</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>literature review</td>
<td>humans and animals</td>
<td>summarize the current literature on the role of lung epithelial cell apoptosis in acute lung injury in children, and related to age/lung development</td>
<td>there is limited knowledge on the effects of lung development on epithelial cell apoptosis during acute lung injury and vice versa</td>
</tr>
<tr>
<td>2</td>
<td>observational; autopsy samples</td>
<td>humans</td>
<td>determine the extent of active caspase-3 immunostaining in lung epithelial cells in children with ARDS</td>
<td>there is a high variability in the extent of classical apoptosis in lung epithelial cells in pediatric ARDS, potentially in part dependent on age</td>
</tr>
<tr>
<td>3</td>
<td>experimental, in vivo and in vitro</td>
<td>mice</td>
<td>determine the contribution of alveolar macrophages to Fas-induced lung inflammation</td>
<td>the lung inflammatory response to Fas activation is not primarily dependent on resident alveolar macrophages and may instead depend on cytokine release by alveolar epithelial cells</td>
</tr>
<tr>
<td>4</td>
<td>observational; epithelial lining fluid, experimental in vitro</td>
<td>humans</td>
<td>determine whether activation of the TRAIL pathway is a potential mechanism of epithelial injury during severe RSV infection in children</td>
<td>severe RSV infection in children is associated with increased concentrations of sTRAIL in the lungs, and sTRAIL induces epithelial injury in vitro</td>
</tr>
<tr>
<td>5</td>
<td>observational; epithelial lining fluid</td>
<td>humans</td>
<td>determine whether the granzyme pathway is activated during severe RSV infection in children</td>
<td>severe RSV infection in children is associated with increased concentrations of active soluble granzymes in the lungs, and these correlate with inflammation</td>
</tr>
<tr>
<td>6</td>
<td>experimental, in vivo</td>
<td>mice</td>
<td>determine whether granzymes contribute to the development of acute lung injury in pneumovirus-infected mice</td>
<td>granzyme A and B-cluster deficiency delays the acute progression of pneumovirus disease by reducing alveolar injury</td>
</tr>
<tr>
<td>7</td>
<td>experimental, in vivo</td>
<td>mice</td>
<td>determine whether mechanical ventilation enhances the host response to pneumovirus infection</td>
<td>mechanical ventilation enhances the activation of inflammatory and caspase cell death pathways in response to pneumovirus infection</td>
</tr>
</tbody>
</table>
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(22) Smith LS, Gharib SA, Martin TR. Transcriptional Analysis of Pulmonary Gene Expression in Adult and Juvenile Mice Treated with Mechanical Ventilation and Lipopolysaccharide. Am J Respir Crit Care Med 179, A2053. 2009.


Appendices
Acute lung injury (ALI), ook wel bekend als acute respiratory distress syndrome (ARDS), is een acute ernstige levensbedreigende longaandoening. ALI/ARDS is een uitgebreide ontstekingsreactie in beide longen die ontstaat door een ziekte van de longen zelf (bijvoorbeeld een longontsteking) of een ziekte ergens anders in het lichaam, zoals bloedvergiftiging of een ernstig trauma. Door de uitgebreide ontsteking ontstaat schade aan het longweefsel en treedt massaal vocht, eiwitten en ontstekingscellen uit de longbloedvaten. Dit is zichtbaar op een röntgenfoto van de longen. Door de ontstekingsreactie wordt de zuurstof opname vanuit de longen in het bloed ernstig bemoeilijkt.

In Nederland worden jaarlijks zo’n 8000 patiënten opgenomen op de intensive care voor kunstmatige beademing vanwege ALI/ARDS. Hoewel ALI/ARDS aanvankelijk vooral bij volwassenen is beschreven kan het ook bij kinderen voorkomen, zij het minder vaak. Ongeveer één derde van de kinderen met ALI/ARDS overlijdt, terwijl bij volwassenen ALI/ARDS in de helft van de gevallen fataal is. Het lijkt er dus op dat kinderen iets minder gevoelig zijn voor deze ziekte. Mogelijk wordt de gevoeligheid beïnvloed door de ontwikkeling en groei van de longen bij jonge kinderen. Van de andere kant zou het kunnen zijn dat de groei van de longen op de lange termijn verstoord wordt door ALI/ARDS op jonge leeftijd. Hiernaar is nog te weinig onderzoek gedaan. Er is momenteel geen specifieke behandeling voor ALI/ARDS; de patiënten kunnen slechts ondersteund worden met kunstmatige beademing en extra zuurstof. Pas als we meer inzicht in de precieze ontstaansmechanismen, maar juist ook de leeftijd afhankelijke verschillen krijgen, kunnen we misschien nieuwe behandelmethoden voor ALI/ARDS vinden.

Dit proefschrift richt zich op de ontstaansmechanismen van schade aan long epitheelcellen bij ALI/ARDS bij kinderen. Epitheelcellen bekleden de binnenkant van de longen en luchtwegen en staan dus direct in contact met de buitenlucht, waar ze zuurstof uit kunnen opnemen. Ze vormen bovendien een barrière tegen bacteriën en virussen, maar zorgen er ook voor dat er geen vocht uit de bloedvaten de longen in lekt. Er zijn tal van aanwijzingen dat ALI/ARDS ontstaat doordat de epitheelcellen in de longen doodgaan. Eén van de manieren waarop cellen kunnen doodgaan is het proces van ‘apoptose’, ook wel cel zelfmoord of geprogrammeerde cel dood genoemd. Apoptose van cellen in het lichaam is normaal gesproken zeer gereguleerd: het zorgt ervoor dat organen hun vorm krijgen, dat cellen die niet meer nuttig zijn opgeruimd worden en dat weefsels niet ‘overgroeien’ (kanker). Er zijn tal van eiwitten en cellulaire paden die apoptose van long epitheelcellen kunnen veroorzaken (hoofdstuk 1). Het blijkt dat deze eiwitten tijdens ALI/ARDS in een verhoogde concentratie aanwezig zijn in het longvocht van patiënten (longvocht is af te nemen via een zogenaamde broncho-alveolaire lavage, BAL). Het identificeren en onderzoeken van deze specifieke eiwitten, zoals FasL, TRAIL en granzymen (hoofdstuk 3-6), is belangrijk voor het vinden van nieuwe aanknopingspunten voor het maken van medicijnen voor ALI/ARDS.
In hoofdstuk 1 stellen wij dat de onbalans van apoptose tijdens ALI/ARDS mogelijk beïnvloed wordt door de ontwikkeling en groei van de longen bij jonge kinderen. In hoofdstuk 2 onderzochten wij longweefsel van kinderen die zijn overleden met ALI/ARDS. We vonden een hoge mate van variabiliteit in de mate van apoptose van long epitheelcellen bij deze kinderen en inderdaad een aanwijzing dat leeftijd hierbij een rol speelt: apoptose in de longen leek minder uitgebreid te zijn bij de jonge kinderen. Dit zou dus kunnen aansluiten bij de bevinding dat jonge kinderen minder gevoelig zijn voor ALI/ARDS.

Aan de andere kant weten we dat er ook specifieke oorzaken van ALI/ARDS op jonge leeftijd zijn, zoals een longinfectie met het respiratoir syncytiaal virus (RSV), waarbij wel een verhoogde mate van apoptose van epitheel cellen aanwezig kan zijn. Om dit verder te onderzoeken hebben we gekeken naar de concentraties van twee van de bovengenoemde eiwitten (TRAIL en granzymen) in de longen van kinderen met een ernstige RSV infectie, in vergelijking met kinderen zonder longaandoening (hoofdstuk 4 en 5). Nadat we hadden aangetoond dat deze eiwitten in hoge mate aanwezig zijn bij kinderen met een RSV infectie, vroegen wij ons af of dit verhoogde longschade zou kunnen veroorzaken. In hoofdstuk 4 laten we zien dat TRAIL inderdaad celdood veroorzaakt van long epitheel cellen die in het laboratorium gekweekt zijn. In hoofdstuk 6 tonen we aan dat granzymen kunnen bijdragen aan de schade en apoptose in de longen van muizen die geïnfecteerd zijn met een ‘knaagdiervariant’ van RSV. Mogelijk spelen deze eiwitten dus een belangrijke rol in het veroorzaken van een ernstige RSV infectie bij kinderen.

Zoals gezegd zijn de behandelmogelijkheden van patiënten met ALI/ARDS tot op heden zeer beperkt. De twee belangrijkste pijlers voor behandeling in de intensieve geneeskunde zijn kunstmatige beademing en zuurstof therapie. Hoewel deze behandelingen levensreddend kunnen zijn, komen er steeds meer aanwijzingen dat ze tegelijkertijd de patiënt kunnen schaden (iatrogene effecten): ze kunnen bijdragen aan het ontstaan van longschade tijdens ALI/ARDS. Deze iatrogene effecten kunnen met name een grote rol spelen bij patiënten met een longontsteking, omdat het is gebleken dat longcellen die door een bacterie zijn geïnfecteerd extra gevoelig zijn voor schade en ontsteking. In hoofdstuk 7 laten wij zien dat kunstmatige beademing echter ook tijdens een virale longontsteking, zoals door RSV, kan bijdragen aan de mate van apoptose en ontsteking in de longen. Hieruit volgt dat kunstmatige beademing van kinderen met een ernstige RSV infectie dus voorzichtig, dat wil zeggen zonder hoge rek van de longen, dient te gebeuren.

Concluderend, ALI/ARDS is een zeer ernstige en levensbedreigende ziekte waar vooralsnog geen specifieke behandeling voor bestaat. Dit proefschrift verbreed het inzicht in de ontstaansmechanismen van ALI/ARDS, specifiek gericht op de rol van apoptose van long epitheelcellen in kinderen. Het draagt bij aan het identificeren van eiwitten die hierbij van belang zijn, met als doel om nieuwe aanknopingspunten voor therapie te vinden.
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CURRICULUM VITAE

Full color illustration section
Figure 1-1. Pathways of caspase-(in)dependent apoptosis in lung epithelial cells mediated by mitochondrial disruption or death receptor stimulation initiated by recruited effector lymphocytes, neutrophils (PMN) or macrophages. Members of the Bcl-2 family including Bcl-2, Bax and Bcl-XL and p53 regulate cytochrome c release from the mitochondria in response to stimuli such as DNA damage, reactive oxygen species (ROS) or calcium overload. Cytochrome c in the cytosol assembles with apoptotic peptidase activating factor 1 (Apaf-1) to activate initiator caspase-9. Adaptor proteins interact through their death domain upon activation of the death receptor pathway by FasL or TRAIL, leading to activation of initiator caspase-8. The mitochondrial and death receptor pathway can interact through Bid. The activation of caspase-8 or caspase-9 leads to the downstream activation of the caspase cascade including effector caspase-3, -6 and -7, resulting in apoptosis. Granzymes delivered to the cytosol by effector lymphocytes can activate caspases and Bid to induce apoptosis. The inhibitor-of-apoptosis proteins (IAPs) can block several caspases thereby inhibiting cell death.

Figure 2-2. A, representative composite images of cytokeratin (blue) and active caspase-3 (red) double staining of lung tissue after unmixing of the individual color spectra, showing co-localization (yellow) (zoomed from 200x magnification images); B, original RedGreenBlue-bright field images (upper left) and composite images: without highlighted co-localization (upper right), active caspase-3 images (lower left) and cytokeratin images (lower right); C, percentage of cytokeratin (ck) positive staining co-localized with positive cleaved caspase-3 staining in alveolar tissue sections of ARDS patients with indirect lung injury (n=4) or direct lung injury (n=12); D, correlation between the percentage of cytokeratin (ck) positive staining co-localized with positive cleaved caspase-3 staining and age (r=0.5, p<0.05).
Figure 2-1. A-C, lung tissue sections of ARDS patients stained with haematoxylin and eosin (magnification 100x) showing changes characteristic of exudative ARDS (*protein-rich alveolar exudates) (A), hyaline membranes (arrow) (B), and marked fibroproliferative changes (C). D-F, Immunohistochemistry for cleaved (active) caspase-3 (magnification 500x) showing the wide range of positive staining in lung tissues sections of patients with ARDS: from widespread positive cells in the alveolar walls (arrows) (D), to almost no positive cells in the alveolar walls (E). Note that the latter picture is comparable to the finding in lung tissue of a patient who died with sepsis, but with normal lung architecture and without clinical ARDS (F).
**Figure 3-1.** Total alveolar macrophages (A) and PMN (B) counts in the BALF of mice 24, 48 or 72 hr after intratracheal instillation of liposomes containing PBS (white) or clodronate (gray) (n=3/group for each time). Panel C and D show representative lung tissue sections from mice studied 24 hr after intratracheal instillation of liposomes containing PBS or clodronate respectively (H&E staining, magnification 400x). Note the absence of macrophages and neutrophils in the lungs from the mouse treated with clodronate liposomes (D).
Figure 3-4. Lung tissue sections from mice treated with intratracheal liposomes containing PBS or clodronate, followed 24 hr later by intratracheal instillations of Jo2 mAb or control IgG mAb, 2.5 µg/g, and studied 18 hr later. The right column shows representative lung tissue sections stained with H&E (400x, insets 200x). The left column shows immunohistochemistry for AM (arrows), using anti mac-2 antibody.
Figure 4-3. A, immunocytochemistry for TRAIL-R1 and -R2 of cytopsins of primary bronchial epithelial cells of children (magnification 500x). B, representative examples of FACS histogram plots of primary bronchial epithelial cells of children stained for membrane bound TRAIL-R1 and -R2. Negative control (grey surface plot) is without primary antibody. C, representative examples of FACS histogram plots for membrane bound TRAIL-R1 and -R2 staining on primary bronchial epithelial cells of children, 3 and 6 days post-infection (dpi) with RSV-A in vitro. Uninfected cells are shown in grey surface plots.
Figure 4-4. A, fold increase in the percentages of annexin V positive cells upon 6 hr exposure to superkillerTRAIL, as compared to cells treated with dilution buffer only. Experiments were performed in duplicates with primary bronchial epithelial cells obtained from three different children. B, example of FACS histogram plots from primary bronchial epithelial cells stained for annexin V upon 6 hr exposure to superkillerTRAIL, as compared to cells treated with dilution buffer only. C, percentages of PI positive primary bronchial epithelial cells from one child upon 48 hr exposure to non cross-linked FLAG-TRAIL with or without M2-anti-FLAG antibody to establish crosslinking. D, percentages of PI positive cells upon 48 hr exposure to non-cross linked FLAG-TRAIL (33 ng/ml) or concentrated BALF from a RSV patient with or without DR5-Fc to neutralize TRAIL signalling. Experiments were performed in duplicates with primary bronchial epithelial cells obtained from two different children. * p <0.05 (paired t-test). Data are shown as bars depicting the mean and standard error. E, representative light microscopy photograph showing the apoptotic morphological appearance of primary bronchial epithelial cells of children in TRAIL cytotoxicity assays (zoomed in 250x magnification). Inset shows normal unexposed control cells (250x magnification).
Figure 6-1. A, gene expression profiles of transcripts encoding GzmA and GzmB in total lung RNA of C57Bl/6 mice (RNA from 5 mice pooled per time point) at time zero and days 1-7, 10, 14, 21 and 28 after inoculation with PVM J3666 (30 pfu). Peak of GzmA and GzmB expression at day 7 (green highlight). B, histogram plots of intracellular GzmA and GzmB expression in BALF CD8+CD3+ cells (CTLs, blue plot), NK1.1+CD3 (NK cells, green plot) and CD8CD3+ (CD8 T lymphocytes, red plot) of PVM-infected mice at day 7 (BALF leukocytes from 3 mice pooled).

Figure 6-6. Representative images of haematoxylin and eosin stained lung tissue from the GzmA+/− GzmB+/− mice and GzmA+/−GzmB−/− mice on day 8 after PVM inoculation (6 x 10^3 copies).
Figure 6-3. A, virus titer in the lung, expressed as number of PVM-sh copies per $10^9$ gapdh copies; B, lung homogenate caspase-3 activity (arbitrary fluorescence units), in the GzmA+/GzmB+/ mice and GzmA+/GzmB+/ mice on day 7 (n=6 per group) and 8 (n=6 per group) after PVM inoculation ($6 \times 10^3$ copies). * p<0.05. Data are shown as box plots depicting the median, interquartile range and full range. C, representative images of cleaved caspase-3 immunohistochemistry in lung tissues of the GzmA+/GzmB+/ mice and GzmA+/GzmB+/ mice on day 8 after PVM inoculation. Note the relative positive staining in alveolar wall cells (arrows) as compared to bronchial epithelial cells in the GzmA+/GzmB+/ mice.
Figure 7-6. Caspase-3 activity in lung homogenates (A); number of TUNEL-positive cells per 12 high-power fields in lung tissue sections (B); BALF soluble FasL concentrations (C) and BALF granzyme B (GrB) concentrations (D) in uninfected mice allowed to breathe spontaneously (SB, n = 6) or subjected to mechanical ventilation (MV, n = 5); and in PVM infected mice allowed to breathe spontaneously (PVM + SB, n = 6) or subjected to mechanical ventilation (PVM + MV, n = 6), * p < 0.05. The cellular distribution of caspase-3 activation is shown in merged differential interference contrast (DIC) and fluorescence images (cleaved caspase-3 in pink, nuclei in blue) from a mouse in the SB group (E); MV group (F), PVM group (G), and PVM + MV group (H). The figures show the presence of signal in cells of the alveolar walls (open arrows) or macrophages (closed arrow).
Figure 7-7. H&E stained lung tissue sections of lung tissue from uninfected mice allowed to breathe spontaneously (SB, A) or subjected to mechanical ventilation (MV, B); and in PVM infected mice allowed to breathe spontaneously (PVM + SB, C) or subjected to mechanical ventilation (PVM + MV, D), 400X magnification. Note the peribronchiolar cellular infiltration in both the PVM + SB and PVM + MV group (arrows). E, mean peak inspiratory pressures in uninfected mice subjected to mechanical ventilation (n = 5) and PVM infected mice subjected to mechanical ventilation (n = 6). Means ± standard error.