Acute lung injury in children: from viral infection and mechanical ventilation to inflammation and apoptosis

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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 (PaO2/FIO2 ratio \( \leq 300 \) mmHg for ALI and PaO2/FIO2 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 4-7. Large, multi-center studies using the AECC criteria have reported mortality rates of 20-35% for pediatric ALI/ARDS 4;5;8, as compared to 40-55% for adults 6;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 10,11, and as such, this adds to the heterogeneity of pediatric ALI/ARDS, making a comparison between children and adults not straight forward.

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 12. Loss of alveolar capillary barrier integrity by epithelial injury is now considered a key event in ALI/ARDS pathogenesis 2;13;14, 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 15;16, 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 (chapter 1). On the other hand, lung development and maturation in young children and infants may affect the (dys)regulation of apoptosis during ALI/ARDS (chapter 1). 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 17. 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 18. Although the precise long term outcome of ALI/ARDS in children that survive is unclear, both restrictive and obstructive functional alterations have been reported 19-21. 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 22;23. 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 (chapter 2). 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 \textit{in vitro} cell culture and \textit{in vivo} mouse experiments. In these, bronchial epithelial cells of children were found susceptible to human recombinant and natural sTRAIL-induced cell death \textit{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 \textit{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 \(48\), lymphocytes and monocytes/macrophages (\textit{chapter 4-6}), are a potential source for (soluble) pro-apoptotic mediators that target lung epithelial cells (\textit{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 \(49,50\). Phagocytosis of apoptotic cells by macrophages inhibits the release of cytokines such as IL-1\(\beta\), IL-8 and TNF\(\alpha\), providing a mechanism that limits tissue inflammation \(51\).

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 \textit{chapter 3} we have presented \textit{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 \textit{in vivo}, consistent with previous findings \(25,27,33,52,53\). Fas ligation activates the key pro-inflammatory transcription factor NF-\(\kappa\)B in macrophages and lung epithelial cells, but how this interaction occurs remains unclear \(54,55\). 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-\(\kappa\)B, but FADD bound to Fas after Fas ligation enables TLR4 pro-inflammatory activity, thereby providing a direct regulatory link between Fas-apoptosis and inflammation \(13,56\). 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 \(57,58\). For example, granzyme A induces the release of pro-inflammatory cytokines such as IL-1\(\beta\), IL-6 and TNF\(\alpha\) 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 in vitro 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\(^{66}\)), and possibly the developmental stage/age of the lungs (chapter 1-2).

Second, we do not know the extent of cell death at which there is critical dysfunction of the lung epithelium in ALI/ARDS \textit{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 \(^{67}\). 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 \(^{32,68-70}\). 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 \(^{68}\). 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 \(^{32,69}\). 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 \(^{71}\).

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 \(^{72,73}\). For example, the application of low tidal volume ventilation has been shown to significantly reduce mortality and inflammation in humans \(^{74,75}\). 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**

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<td>determine the extent of active caspase-3 immunostaining in lung epithelial cells in children with ARDS</td>
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<td>determine whether activation of the TRAIL pathway is a potential mechanism of epithelial injury during severe RSV infection in children</td>
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<td>5</td>
<td>observational; epithelial lining fluid</td>
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<td>determine whether the granzyme pathway is activated during severe RSV infection in children</td>
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<td>7</td>
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REFERENCES


