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TNF-Induced Death Signaling Triggers Alveolar Epithelial Dysfunction in Acute Lung Injury

Brijesh V. Patel, Michael R. Wilson, Kieran P. O’Dea, and Masao Takata

The ability of the alveolar epithelium to prevent and resolve pulmonary edema is a crucial determinant of morbidity and mortality in acute lung injury (ALI). TNF has been implicated in ALI pathogenesis, but the precise mechanisms remain undetermined. We evaluated the role of TNF signaling in pulmonary edema formation in a clinically relevant mouse model of ALI induced by acid aspiration and investigated the effects of TNF p55 receptor deletion, caspase-8 inhibition, and alveolar macrophage depletion on alveolar epithelial function. We found that TNF plays a central role in the development of pulmonary edema in ALI through activation of p55-mediated death signaling, rather than through previously well-characterized p55-mediated proinflammatory signaling. Acid aspiration produced pulmonary edema with significant alveolar epithelial dysfunction, as determined by alveolar fluid clearance (AFC) and intra-alveolar levels of the receptor for advanced glycation end-products. The impairment of AFC was strongly correlated with lung caspase-8 activation, which was localized to type I alveolar epithelial cells by flow cytometric analysis. p55-deficient mice displayed markedly attenuated injury, with improved AFC and reduced caspase-8 activity but no differences in downstream cytokine/chemokine production and neutrophil recruitment. Caspase-8 inhibition significantly improved AFC and oxygenation, whereas depletion of alveolar macrophages attenuated epithelial dysfunction with reduced TNF production and caspase-8 activity. These results provide in vivo evidence for a novel role for TNF p55 receptor-mediated caspase-8 signaling, without substantial apoptotic cell death, in triggering alveolar epithelial dysfunction and determining the early pathophysiology of ALI. Blockade of TNF-induced death signaling may provide an effective early-phase strategy for ALI. The Journal of Immunology, 2013, 190: 4274–4282.

Acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) is a major cause of morbidity and mortality in critical care, and no treatment exists beyond supportive therapies (1, 2). ALI is characterized first by a severe disruption of the alveolar–capillary barrier, leading to life-threatening pulmonary edema, and second by intense pulmonary inflammation involving recruitment of blood leukocytes. Although leukocytes further aggravate damage to the alveolar–capillary barrier (3–5), edema can develop in the absence of neutrophils (6, 7) and manifest within minutes to hours of an initiating insult, well before substantial leukocyte infiltration into the alveolar space (8, 9). The alveolar epithelium plays a central role in providing the greatest resistance to edema formation (10), and its disruption significantly promotes the progression of ALI through both increased barrier permeability and decreased alveolar fluid clearance (AFC). Alveolar epithelial dysfunction, as quantitatively assessed by a decreased AFC rate, significantly influences mortality as a result of ARDS through diminished resolution of edema (11).

It has been shown that alveolar epithelial injury in ALI is associated with apoptosis of epithelial cells (12). Apoptosis proceeds through two main pathways: the intrinsic pathway, which is mediated through release of cytochrome c from mitochondria, leading to activation of caspase-9, and the extrinsic pathway, which is initiated by the ligation of death receptors on the cell surface, such as Fas and TNFR1 (p55), resulting in activation of “death signaling” involving caspase-8. A number of studies have identified mechanisms through which the Fas ligand (FasL)/Fas interaction mediates epithelial cell apoptosis during ALI (13–15); in some reports, TNF-induced apoptosis has been implicated in ALI, although its significance has not been fully elucidated (16). Importantly, the majority of these studies focus on the relatively late phases of ALI, and it is not certain whether apoptosis has any direct pathophysiological impact on the early development of this syndrome. Moreover, only a very small number of actual dead cells have been observed in lung tissue, for example, up to a maximum of 10% (which included many apoptotic neutrophils) of total lung cells reported in autopsied ARDS lungs (13). Such a low degree of epithelial cell loss does not seem sufficient to explain the significant deterioration in lung function observed in these experimental and clinical studies. The potential influence of death signaling per se on the functional status of the alveolar epithelium, before actual cell death that is the final event in the apoptotic process takes place, has not been investigated.

In this study, we sought to investigate the role of TNF, an early-phase proinflammatory cytokine, as well as a death ligand, in pulmonary edema formation during ALI. TNF has often been implicated in the pathogenesis of ALI (17), but therapies to block its actions have been applied to critical illness in a rather premature manner, without a proper understanding of the precise mechanisms through which it functions. In this article, we present...
important in vivo evidence that alveolar macrophage–derived TNF plays a crucial role in triggering alveolar epithelial dysfunction, leading to pulmonary edema, through activation of its p55 receptor–mediated death signaling involving caspase-8 rather than through previously well characterized proinflammatory signaling. Our findings reveal a novel concept that TNF-mediated death signaling per se, without producing significant apoptotic cell death, determines the functional derangement of the alveolar epithelium and early pathophysiology of ALI. This offers important insights into potential new treatments for ALI targeting death signaling.

Materials and Methods

Model of experimental lung injury

All protocols were approved by the Ethical Review Board of Imperial College London and carried out under the authority of the UK Home Office in accordance with the Animals (Scientific Procedures) Act 1986, U.K. We used male wild-type (WT) C57BL6 (Charles River) and TNF p55 receptor knockout (p55−/−) mice (The Jackson Laboratory). The surgical preparation has been previously described in detail (18). In brief, mice were anesthetized by i.p. injection of ketamine (80 mg/kg) and xylazine (8 mg/kg) and underwent tracheostomy and noninjurious low tidal volume ventilation (8–9 ml/kg tidal volume, 2.5 cm H2O positive end-expiratory pressure, 120 breaths/min and FiO2 of 1.0) using a custom-made mouse ventilator–pulmonary function testing system. The left carotid artery was cannulated for monitoring arterial blood pressure and blood gases, as well as for saline infusion (0.4 ml/h). Once instrumentation was complete, baseline blood gases, peak inspiratory pressure, and respiratory mechanics were recorded. Subsequently, hydrochloric acid was instilled via the tracheostomy tube using a fine catheter, and ventilation continued for 90 or 180 min, depending on end points measured. After pilot experiments titrating the dose and concentration, we were able to create a robust, reproducible lung injury over 3 h using 50 μl 0.15 M hydrochloric acid (pH 1.5).

Physiological measurements

Airway pressure, gas flow, and mean blood pressure were continuously monitored throughout the experimental protocol, whereas respiratory mechanics and arterial blood gases were assessed at predetermined intervals. Respiratory system elastance was measured using the end-inspiratory occlusion technique, as described previously (19). Sustained inflation of 30 cm H2O for 5 s was performed every 30 min to maintain alveolar recruitment.

Lung edema and bronchoalveolar lavage fluid analyses

At the end of the 3-h experimental protocol, mice were sacrificed, and the left lung was removed, weighed, and dried at 60˚C for 24 h for wet/dry weight analysis. Bronchoalveolar lavage of the right lung was performed with 400 μl saline, as described previously (18), and the samples were centrifuged at 1500 rpm. Protein levels in bronchoalveolar lavage fluid (BALF) were quantified by Bradford assay (Bio-Rad), and BALF levels of IL-6, TNF, KC, and MIP-2 were determined using ELISA assay kits (R&D Systems).

Lung-permeability analyses

In some animals, Alexa Fluor 594–conjugated albumin (Invitrogen) was injected via the external jugular vein 30 min before the end of the 3-h protocol. The alveolar–capillary barrier permeability index was calculated as the ratio of BALF/plasma fluorescence (Biotek), as described previously (20).

Lung leukocyte quantification

In a separate series of experiments, following termination, left lungs were processed for histological staining (see below), whereas right lungs were used to prepare lung single-cell suspensions, as described previously (4, 21). In brief, lungs were mechanically disrupted using a GentleMACS tissue dissociator (Miltenyi Biotec) for 1 min in Intracellular fixative (eBioscience). They were then sieved through a 40-μm filter in a flow cytometry wash buffer (PBS with 2% FCS, 0.1% sodium azide, and 5 mM EDTA). Cells were stained in the dark for 30 min with fluorophore-conjugated anti-mouse Abs to CD45 (clone 30-F11; BioLegend), CD11b (M1/70; BioLegend), NK1.1 (PK136; BioLegend), Gr-1 (RB6-8C5; BioLegend), and Ly-6C (AL-21, Becton Dickinson Biosciences). Samples were analyzed using a CyAn ADP flow cytometer (Beckman Coulter) after the addition of Acrichrome counting beads (Invitrogen). Analysis was performed using FlowJo software (TreeStar).

Histology

Left lungs of this group of animals underwent intratracheal instillation with 4% paraformaldehyde at a transpulmonary pressure of 15 cm H2O. They were subjected to paraffin embedding, and 5-μm sections were attained. Sections were stained with H&E (Sigma-Aldrich) for examination. In addition, TUNEL staining was carried out to detect apoptotic DNA damage using the TACS.XL diaminobenzidine (DAB) in situ apoptosis detection set ( Trevigen), per the manufacturer’s instructions. Apoptosis index was quantified on 20 random sections (at 400X magnification) of the left lung/mouse by an investigator blinded to the groups (16). TUNEL+ cells were counted as those with a distinct DAB+ nucleus within the alveolar wall. Images were acquired using a BX-60 light microscope (Olympus) and a digital AxioCam camera using KS300 v3.0 software (both from Zeiss).

Measurement of caspase-8 activity

A third group of animals underwent acid aspiration for functional determination of AFC. This was measured using an ex vivo, in situ set-up, as described previously in detail (22). In brief, 90 min post-acid instillation, animals were sacrificed and exsanguinated. Mice then underwent intratracheal instillation with 700 μl of an iso-osmolar medium containing 5% low-endotoxin BSA (Sigma-Aldrich) and 50 μg/ml fluorescent Alexa Fluor 594–conjugated BSA (Invitrogen), followed by removal of acid at 30 min post-acid instillation.书房上酸的系统被放置在30分钟内，然后进行实验。30分钟后的样本(30m sample)被放在了液体中。细胞中的这个氟化物-荧光素标记的化合物会扩散出细胞。在进一步清洗后，放到了一个冰水池中进行正位空气压力系统在氧气饱和度为80%的条件下，以36.5 ~ 38°C的温度存储。30分钟后，安装了一个新的肺切除术，以诱导肺的切除，从而在肺泡上形成一个细胞丝。然后，这些样本被测量了平均细胞数(PerkinElmer)。使用流式细胞计数器(流式细胞计数器)来测量细胞数。为了确定细胞对AFC的反应率。

Measurement of caspase-8 activation

Caspase-8 activity was measured in lung homogenates using a Caspase-8/FLICE Fluorometric Assay Kit (BioVision), per the manufacturer’s instructions. The left lungs of animals (post-AFC measurement) underwent a standardized homogenization in the cell lysis buffer provided, using a hand-held homogenizer for 30 s.

Localization of caspase-8 activation

In the fourth group of animals, localization of caspase-8 activation within the lung was evaluated in WT mice using flow cytometry. Lung single-cell suspensions were prepared from lungs of uninjured and injured animals at 90 min after acid instillation. To efficiently recover epithelial and endothelial cells, we used a modification of a previously published tissue-digestion protocol (21, 23–25). Lungs were instilled with 1 ml DMEM containing 1 mg/ml sterile filtered Dispase (Invitrogen) via the tracheostomy and placed in the same Dispase solution for 30 min at room temperature. Lungs were subsequently placed in ice-cold sterile DMEM/2.5% HEPES with 0.01% DNase (Roche), and the parenchymal tissue was separated from the bronchial tree and gently minced. This suspension of the distal lung tissues was passed through a 40-μm filter, washed, and reconstituted with ice-cold DMEM/2.5% HEPES. Cells were incubated with 3.33 μl FAM-IETD-FMK caspase-8 reagent (Immunochemistry) for 60 min at 37°C in the dark, per the manufacturer’s instructions, to enable this fluorochrome-conjugated caspase-8 inhibitor–based compound to permeate the cells. The reagent binds specifically to activated intracellular caspase-8, which can be detected later in individual cells by flow cytometry (in the FITC channel). Cells were then washed, resuspended in DMEM/2.5% HEPES, and incubated for 15 min at 37°C, allowing any unbound caspase-8 reagent to diffuse out of the cells. After a further wash, cells were stained with fluorophore-conjugated anti-mouse Abs to pan-endothelial marker CD31 (clone MEC 13.3; BioLegend), the pan-leukocyte marker CD45, the pan-epithelial marker epithelial cell adhesion molecule (G8.8; eBioscience), and the type 1 alveolar epithelial cell (AEC) marker–type 1 cell α protein (T1alpha, 8.1.1; eBioscience), and analyzed by flow cytometry as described above. Of note, lung cell suspensions were unfixed and kept on ice throughout the protocol, except during the incubation/wash steps for the FAM-IETD-FMK caspase-8 reagent binding.
In vivo caspase-8 inhibition

In the fifth series of experiments, 4 mg/kg of the selective caspase-8 inhibitor Z-VDAD-DEVD-FMK (Becton Dickinson Biosciences), or DMSO as the vehicle, was administered i.v. (via the external jugular) to WT mice 5 min before intratracheal acid instillation, and AFC and oxygenation were measured at 90 min.

Depletion of resident alveolar macrophages

In the final series of experiments, WT mice were anesthetized 48 h prior to acid aspiration and underwent laryngoscopy, as described previously (22), for intratracheal instillation of 75 μl clodronate or PBS encapsulated into liposomes (Encapsula NanoSciences) to deplete resident alveolar macrophages. We used a previously described flow cytometric identification of resident alveolar macrophages to evaluate the extent of their depletion (9). In brief, lung cell homogenates were prepared as described above and stained in the dark for 30 min with fluorochrome-conjugated anti-mouse Abs to CD45, F4/80 (C1.3; BioLegend), CD11b, and CD11c (N418; eBioscience). Alveolar macrophages were identified as CD45hiF4/80hi CD11chi and CD11blo. AFC was determined as described above, and soluble levels of TNF, FasL, and RAGE were measured by ELISA (R&D Systems) in t30 samples.

Statistics

Data are expressed as means ± SD or median ± interquartile range (if nonparametric) and analyzed using SPSS version 20 (IBM). The model assumption of normality of residuals was assessed by QQ plot and the Shapiro–Wilk test. Statistical analyses of data were made using either a two-tailed Student t test or one-way ANOVA with Bonferroni tests for multiple comparisons; Mann–Whitney/Kruskal–Wallis tests were used for nonparametric data. Time courses were analyzed using a t test of final end point values. We used Pearson correlation coefficients to test the relationships between continuous variables. Statistical significance was defined as p < 0.05.

Results

TNF p55 receptor plays a crucial role in the development of pulmonary edema in ALI

Acid instillation induced an immediate spike in respiratory system elastance (Fig. 1A) due entirely to the presence of fluid within the airways, because saline instillation induced the same initial elastance change, which rapidly returned to baseline as the fluid was absorbed. In acid-instilled WT animals, elastance remained high after this initial spike and started to increase further toward the end of the 3-h protocol, indicating formation of pulmonary edema. In sharp contrast, p55+/− mice displayed a steady recovery in elastance from the initial spike toward the baseline, in a similar fashion to saline-instilled WT animals. The p55+/− strain had significantly improved arterial oxygenation compared with WT animals (Fig. 1B). At 3 h, acid instillation in WT animals produced considerable increases in lung wet/dry weight ratio (Fig. 1C), total protein levels in BALF (Fig. 1D), and alveolar–capillary barrier permeability index (Fig. 1E), all of which were markedly attenuated in p55−/− mice. This effect of TNF p55 receptor signaling on alveolar edema development was apparent as early as 60–90 min after injury was initiated, by which point the elastance curves had clearly diverged.

TNF-induced early development of pulmonary edema is not due to enhanced downstream proinflammatory responses

To investigate the role of TNF-induced downstream proinflammatory signaling in this model, we studied levels of cytokines/chemokines and leukocyte recruitment within the lung at 3 h. We first confirmed a clear upregulation of soluble TNF (ligand) itself in BALF in the WT and p55−/− strains at comparable levels (Fig. 2A). Acid aspiration led to significant increases in alveolar IL-6, KC, and MIP-2 (Fig. 2B–D), but there were no differences between WT and p55−/− strains.

Cytometric analysis showed minimal neutrophilia in BALF in all groups (percentage neutrophils in BALF cells: WT uninjured, undetectable: WT acid, 3.3 ± 1.7%; p55−/− acid, 4.1 ± 2.4%). However, there were significant increases in lung-sequestered neutrophils and inflammatory (Ly-6C+) monocytes (Fig. 2E). Despite detecting only minimal numbers of resident alveolar macrophages, and alveolar edema) were apparent between the two strains, whereas leukocyte infiltration seemed to be similar (Fig. 2F).

Acid-induced lung injury produces early activation of TNF p55 receptor–mediated death signaling

Based on the above findings, we hypothesized that TNF p55 receptor–mediated activation of death signaling, rather than proinflammatory signaling, may play an important role in the development of ALI. We examined lung sections for nuclear changes suggestive of late-stage cellular apoptosis (Fig. 3A–D). Acid instillation produced a subtle increase in the number of TUNEL+ cells within the alveoli in WT animals at 3 h, which was reduced in p55−/− mice (Fig. 3E). Despite detecting only minimal numbers of dead cells, we found that lung caspase-8 activity was upregulated considerably in WT animals; this was markedly attenuated in p55−/− mice (Fig. 3F). Examination of the time course...
of caspase-8 activation in WT mice revealed that the activity was increased significantly, even at 90 min (Fig. 3G). Because 90 min was also the time at which physiological differences between WT and p55

The observed rapid development of pulmonary edema with increased barrier permeability is the prominent feature of ALI/ARDS, implying damage to the alveolar epithelium and/or pulmonary endothelium. The results of the caspase-8 localization analysis confirmed that the impact of TNF p55 death signaling is focused on the type 1 AECs. Because epithelial injury is a critical determinant of ALI pathophysiology, we specifically evaluated the involvement of the alveolar epithelium by also measuring AFC to assess its physiological function and measuring BALF RAGE, a biomarker for type 1 AEC injury (27).

TNF p55 receptor signaling triggers early alveolar epithelial dysfunction during lung injury

The observed rapid development of pulmonary edema with increased barrier permeability is the prominent feature of ALI/ARDS, implying damage to the alveolar epithelium and/or pulmonary endothelium. The results of the caspase-8 localization analysis confirmed that the impact of TNF p55 death signaling is focused on the type 1 AECs. Because epithelial injury is a critical determinant of ALI pathophysiology, we specifically evaluated the involvement of the alveolar epithelium by also measuring AFC to assess its physiological function and measuring BALF RAGE, a biomarker for type 1 AEC injury (27).

In WT mice, acid aspiration produced a significant (49%) deterioration of AFC (Fig. 5A) and upregulation of BALF RAGE (Fig. 5B) compared with uninjured animals. There were strong inverse correlations between AFC and BALF RAGE levels (Fig. 5C; Pearson $r = -0.901; p < 0.0001$) and between AFC and re-
Physiological dysfunction. This index was further reduced in p55 treated with a nuclease enzyme to promote DNA strand breaks, illustrating the maximum number of alveolar nuclei that could be stained TUNEL+ (*). The upregulation of caspase-8 activity is an early event in the pathogenesis of acid-induced lung injury (*). The resultant caspase-8 activation in type 1 AECs dictates lung epithelial injury and dysfunction, as assessed by AFC and BALF soluble RAGE levels. Our findings support a novel concept relating TNF-mediated "death signaling" to regulating alveolar epithelial dysfunction and development of pulmonary edema during the early phase of ALI. Using an in vivo mouse model of acid-induced ALI, we found that alveolar macrophage-derived TNF promotes pulmonary edema formation, specifically through activation of the TNF p55 receptor and its downstream death signaling. The resultant caspase-8 activation in type 1 AECs dictates lung epithelial injury and dysfunction, as assessed by AFC and BALF soluble RAGE levels. Our findings support a novel concept that TNF p55 receptor-mediated death signaling per se produces significant dysfunction in the alveolar epithelium, and this effect, rather than actual apoptotic cell death, determines the pathophysiology and physiological derangement in early ALI.

In the absence of actual cell death, we sought to clarify the causal link between activation of death signaling per se and alveolar epithelial dysfunction, both induced by TNF p55 receptor ligation. Hence, we administered a caspase-8–specific inhibitor Z-VAD-IETD (4 mg/kg in DMSO, i.v.) just before acid instillation and measured AFC at 90 min in WT animals. We found a marked improvement in AFC (Fig. 6A) and oxygenation (Fig. 6B) in mice treated with the inhibitor compared with vehicle (DMSO)-treated controls. Furthermore, lung caspase-8 activity showed a strong inverse correlation with AFC (Pearson $r = -0.843; p < 0.0001$) (Fig. 6C), implying that caspase-8 activation has a fundamental influence upon epithelial function in early ALI.

**Caspase-8 activation dictates the functional ability of the alveolar epithelium to clear fluid**

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Alveolar macrophage–derived TNF is required for caspase-8–induced alveolar epithelial dysfunction

Resident alveolar macrophages are prominent early producers of TNF, and their depletion attenuates acid-induced ALI (28). To investigate their contribution to the development of alveolar epithelial dysfunction, we depleted their population to $\sim 10\%$ in WT mice by intratracheally administering clodronate liposomes (48 h prior to acid instillation) (Fig. 7A). The increase in BALF TNF levels observed at 90 min after acid instillation in control animals (pretreated with PBS liposomes) was, as expected, virtually abolished in alveolar macrophage–depleted mice (pretreated with clodronate liposomes) (Fig. 7B).

This depletion of alveolar macrophages markedly attenuated the activation of caspase-8 within the lung (Fig. 7C) to levels similar to those found in the p55$^{-/}$ mice. Interestingly, we found no detectable soluble FasL in BALF of acid-injured animals, suggesting that death signaling and caspase-8 activity at this time point (90 min) were driven predominantly by TNF. Macrophage depletion also produced a significant reduction in BALF RAGE levels (Fig. 7D) and substantial (1.8-fold) improvement in AFC (Fig. 7E), ultimately leading to improved oxygenation (Fig. 7F). Once again, there was a strong inverse correlation between caspase-8 activity and AFC (Fig. 7G; Pearson $r = -0.845; p < 0.0001$) and between RAGE and AFC (Fig. 7H; Pearson $r = -0.894; p < 0.0001$).

**Discussion**

To our knowledge, this is the first study to demonstrate a crucial role for TNF-mediated “death signaling” in regulating alveolar epithelial dysfunction and development of pulmonary edema during the early phase of ALI. Using an in vivo mouse model of acid-induced ALI, we found that alveolar macrophage–derived TNF promotes pulmonary edema formation, specifically through activation of the TNF p55 receptor and its downstream death signaling. The resultant caspase-8 activation in type 1 AECs dictates lung epithelial injury and dysfunction, as assessed by AFC and BALF soluble RAGE levels. Our findings support a novel concept that TNF p55 receptor–mediated death signaling per se produces significant dysfunction in the alveolar epithelium, and this effect, rather than actual apoptotic cell death, determines the pathophysiology and physiological derangement in early ALI.

Using p55-deficient animals, we found that TNF, through its p55 receptor, plays a major role in the early alveolar epithelial injury following acid aspiration. TNF signals through two cell surface receptors: TNF receptor 1 (p55, TNFRSF1a, CD120a) and TNF receptor 2 (p75, TNFRSF1B, CD120b). The majority of responses to TNF are mediated via p55 signaling, although it has become clear that p75 signaling may have independent, even opposing, effects. The current study focuses on the p55 receptor because in cases in which such differential signaling has been
demonstrated, p55 is universally the “deleterious” receptor (20, 29, 30) and, thus, is much more amenable to the development of pharmacotherapies (31). Ligand binding to p55 allows the assembly of TNFR complex I on the cell membrane, leading to NF-κB– and MAPK-mediated gene transcription (32). This is at the heart of the classic roles of TNF “proinflammatory” signaling in promoting the production of downstream inflammatory cytokines/chemokines and migration of leukocytes (33). However, our results indicate that the injurious effect of p55 signaling on alveolar–capillary barrier dysfunction is not dependent on the classic inflammatory consequences of TNF, consistent with our previous report using a mouse model of ventilator-induced lung injury (20); this suggests that the current findings are not model specific.

The alternative pathway to this proinflammatory signaling is so-called “death signaling,” which involves internalization of complex I, leading to the formation of TNFR complex II and subsequent recruitment of the death domain proteins TNFR-associated death domain and Fas-associated death domain (34). Crucially, death signaling is only induced through the p55 TNFR and not the p75 receptor. The mechanisms behind the decision-making process between complex I and II remain unclear, although there is evidence that the ability of complex I to activate NF-κB may shift the cells in an antiapoptotic proinflammatory direction (35). Complex II helps to recruit and activate caspase-8, a key modulator of death signaling, which ultimately activates executioner caspases, such as caspase-3, promoting cell death. In this study, we found early activation of p55 receptor–mediated death signaling involving caspase-8 after acid aspiration and obtained clear in vivo evidence that this death signaling, rather than classical proinflammatory cascades, plays a crucial role in determining assembly of TNFR complex I on the cell membrane, leading to NF-κB– and MAPK-mediated gene transcription (32). This is at the heart of the classic roles of TNF “proinflammatory” signaling in promoting the production of downstream inflammatory cytokines/chemokines and migration of leukocytes (33). However, our results indicate that the injurious effect of p55 signaling on alveolar–capillary barrier dysfunction is not dependent on the classic inflammatory consequences of TNF, consistent with our previous report using a mouse model of ventilator-induced lung injury (20); this suggests that the current findings are not model specific.

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physiological dysfunction of the epithelium, as assessed by impaired AFC and elevated BALF soluble RAGE levels.

It is important to note that alveolar epithelial dysfunction manifested in this study long before significant apoptotic cell death took place. Even by 3 h, TUNEL+ cell numbers were very limited, indicating that the substantial lung dysfunction observed cannot be attributed simply to epithelial cell loss. Although efficient clearance of dead cells by macrophages, as seen in reports studying in vivo phagocytosis of exogenously applied apoptotic bodies (36), may explain this scarcity, it is not likely that both the end-stage apoptosis and efferocytosis processes take place at this early time point (90–180 min after injury). Furthermore, there were no histological signs of disruption of alveolar structure or proliferative response that are expected to occur following apoptosis and removal of structural cells, such as alveolar epithelium. (37). As described before, the extent of morphological apoptosis (i.e., TUNEL+ cells) in ARDS nonsurvivors is, in general, limited (i.e., up to 10%, which includes apoptotic neutrophils) (13), and the majority of animal models of ALI at the peak of injury show minimal levels of TUNEL+ cells (38, 39). This situation is very similar to the one in chronic heart failure: despite the convincing evidence for apoptosis involvement in disease progression, the majority of cardiac myocytes do not show nuclear damage (i.e., are not yet dead) and, with only changes to cytoplasmic apoptotic signaling, still present significant systolic dysfunction (40).

These discrepancies between the extent of “terminal” apoptotic cell death and physiological indices of cell/organ dysfunction have not attracted proper attention in ALI research. The present study provides clear evidence that caspase-8 activation within the type 1 alveolar epithelium determines the degree of impairment in AFC and oxygenation, supporting a new concept that TNF-induced death signaling itself, rather than the number of dead cells, determines alveolar epithelial dysfunction and injury in the early phase of ALI.

A number of mediators have been shown to influence AFC in animal and clinical studies (41, 42). TNF was proposed to have an opposing impact on AFC, with receptor binding inducing inhibition.
and its lectin-like domain promoting reabsorption (43). To our knowledge, this study is the first to imply that death signaling has a direct influence on AFC. However, the mechanism by which this occurs is unclear. One possibility is that localization of activated caspase-8 to the mitochondrial membrane (44) impedes mitochondrial function and reduces ATP production necessary for basolateral sodium potassium ATPase transporter function. Alternatively, TNF may downregulate apical epithelial Na channel expression or function, as observed in type 2 pneumocytes in vitro (45), through further downstream consequences of death signaling, for example, phosphorylation of myosin L chain kinase, which has been shown to induce dynamic cell membrane blebbing through increased cytoskeletal contractility (46). Indeed, hypoxia was shown to induce alveolar epithelial cytoskeletal disruption and reduce epithelial Na channel expression, which are recovered by a pan-caspase inhibitor (47), suggesting that death signaling may affect ion channel expression/activity. The mechanisms behind this novel role for TNF death signaling in regulating alveolar fluid dynamics remain to be fully explored.

The results of alveolar macrophage–depletion experiments, which confirmed the previously reported importance of alveolar macrophages in acid-induced ALI (28), showed decreased TNF production as expected, along with attenuated lung caspase-8 activation, reduced BALF RAGE levels, improved AFC, and improved physiological indices (i.e., oxygenation). Importantly, in acid-instilled PBS liposome-treated mice, we could not detect soluble FasL within the alveolar space, whereas a substantial increase in soluble TNF was detected. However, there is a possibility that membrane-bound FasL activates Fas on epithelial cells, leading to capsase-8 activation. This may explain why p55 receptor–knockout animals show only a 60% reduction in caspase-8 activation compared with WT animals. Although we have not completely excluded the involvement of cell surface FasL from alveolar macrophages, Fas-induced lung injury (using the anti-Jo2 Ab) was shown to worsen, rather than to improve, following alveolar macrophage depletion (48). Of note, the majority of studies report alveolar FasL/Fas activation at later time points (>4–6 h), potentially as a consequence of FasL release by blood-derived leukocytes (14, 49, 50).

There is growing evidence that apoptosis may not be as simple and straightforward a process as once considered (51): an injured cell may enter a state of “apoptotic limbs” (i.e., always on the edge of death), depending on, for instance, a threshold of initial caspase activation (52) or the availability of cellular ATP for apoptosis to follow through to completion (53). Prior to this threshold, cell death may be reversible; however, once a critical threshold is reached, nuclear damage is inevitable, ultimately leading to irreversible cell death. In ALI, this may be dependent on the extent to which death signaling is initially activated by early-phase ligands, such as TNF from alveolar macrophages, and subsequently perpetuated by later-phase mediators, such as Fas driven by infiltrating leukocytes. These considerations suggest new early-phase therapeutic strategies for ALI/ARDS—targeting early death signaling, for example, specific blockade of p55 receptor (31) or caspase-8 activation, which may result in recovery from alveolar epithelial dysfunction and injury.

There are some important caveats to our work. Although the acid-ascension model is a widely used model of ALI and often is quoted as the most clinically relevant among the currently available ALI models (54, 55), it has many limitations: for instance, it uses acid solutions with much lower pH than that of aspirated gastric contents, and clinical aspiration includes other particulate matter, such as food and bacterial products. Thus, direct extrapolation from this model to clinical ARDS should be done with caution. However, the aim of our model was to produce significant physiological deterioration (acquiring oxygenation indices similar to clinical ARDS), enabling us to assess the mechanisms of alveolar epithelial dysfunction. Moreover, we showed previously that deletion of the p55 receptor leads to a similar protective effect, seemingly also independent of downstream inflammation, in a model of stretch-induced lung injury (20). Collectively, these findings strongly suggest that TNF-mediated death signaling has wider implications in the pathogenesis of epithelial dysfunction in lung injury of various etiologies.

In conclusion, we present a critical role for early intrapulmonary death signaling and alveolar epithelial dysfunction in ALI, mediated by alveolar macrophage–derived TNF producing caspase-8 activation through its p55 receptor. This activation of death signaling within AECs, rather than cellular loss through completed apoptosis, critically regulates the ability of the alveolar epithelium to resolve pulmonary edema through AFC. The work provides a coherent link to a number of unresolved paradigms implicated in the early pathogenesis of ALI: integrating the injurious role of alveolar macrophages, TNF, epithelial injury and apoptosis, and AFC. Finally, the results provide a basis to consider translational potential for novel strategies targeting alveolar epithelial death signaling and dysfunction to treat ALI/ARDS.

Disclosures
The authors have no financial conflicts of interest.

References


32. Wajant, H., K. Pfizenmaier, and P. Scheurich. 2003. Tumor necrosis factor alpha (TNF-alpha)-induced cell adhesion to human endothelial cells is under dominant control of one TNF receptor type, TNF-R55.
