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Synergy between acid and endotoxin in an experimental model of aspiration-related lung injury progression

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Tetenev K, Cloutier ME, von Reyn JA, Ather JL, Candon J, Allen GB. Synergy between acid and endotoxin in an experimental model of aspiration-related lung injury progression. *Am J Physiol Lung Cell Mol Physiol* 309: L1103–L1111, 2015. First published September 25, 2015; doi:10.1152/ajplung.00197.2014.—Aspiration is a common cause of lung injury, but it is unclear why some cases are self-limited while others progress to acute respiratory distress syndrome (ARDS). Sporadic exposure to more than one insult could account for this variable progression. We investigated whether synergy between airway acid and endotoxin (LPS) amplifies injury severity in mice and whether LPS levels in human patients could corroborate our experimental findings. C57BL/6 mice aspirated acid (pH 1.3) or normal saline (NS), followed by LPS aerosol or nothing. Bronchoalveolar lavage fluid (BALF) was obtained 2 to 49 h later. Mice were injected with FITC-dextran 25 h after aspiration and connected to a ventilator, and lung elastance (H) measured periodically following deep inflation (DI). Endotracheal and gastric aspirates were also collected from patients in the intensive care unit and assayed for pH and LPS. Lung instability (ΔH following DI) and pressure-volume hysteresis in *acid*- or *LPS*-exposed mice was greater than in controls but markedly greater in the combined *acid/LPS* group. BALF neutrophils, cytokines, protein, and FITC-dextran in the *acid/LPS mice* were geometrically higher than all other groups. BALF from *acid-only* mice markedly amplified LPS-induced TNF-α production in cultured macrophages. Human subjects had variable endotracheal LPS levels with the highest burden in those at higher risk of aspiration. Acid aspiration amplifies LPS signaling in mice to disrupt barrier function and lung mechanics in synergy. High variation in airway LPS and greater airway LPS burden in patients at higher risk of aspiration could help explain the sporadic progression of aspiration to ARDS.

lung mechanics; respiratory impedance; forced oscillations; acid aspiration; endotoxin; acute lung injury; ARDS

ACUTE RESPIRATORY DISTRESS syndrome (ARDS) is a potentially lethal form of noncardiogenic pulmonary edema and acute hypoxemic respiratory failure with a mortality of up to 60%, depending on age (31). Numerous different direct and indirect insults such as pneumonia, sepsis, and aspiration of gastric contents can cause ARDS (10, 13, 39). In the case of aspiration, the clinical course is highly variable, with some patients developing a mild, self-limited pneumonitis and others progressing to fulminate ARDS (22, 41). The reasons behind this variable course remain a complete mystery, but one potential explanation is an unrecognized sporadic exposure to more than one insult at the time of aspiration. In this regard, so-called "two-hit" models of ARDS have shown how two different insults can lead to amplified injury when occurring in combination (5, 25, 26). Such sporadic exposure and synergy could explain why a seemingly random cohort of aspiration pneumonitis cases progress to ARDS while others do not.

The Gram-negative bacterial cell-wall constituent lipopolysaccharide (LPS) is an endotoxin so ubiquitous in our environment that small amounts can be routinely detected within the lung lavage fluid and plasma of critically ill patients (23, 28). However, detectable levels of airway endotoxin are not sufficient to trigger certain progression to ARDS (24, 36). In addition to frequently documented bacterial colonization of the stomach and airways in mechanically ventilated patients (6, 14, 34), we now know that aspiration of gastric contents into the airways often occurs within hours of tracheal intubation (33, 35). As such, in addition to the initial caustic injury incurred from acidic aspirate (21), LPS is a highly plausible candidate for an adjuvant inflammatory insult to promote the progression of aspiration-induced injury.

Despite our current understanding of ARDS pathogenesis and the complex signaling that follows both airway endotoxin exposure and acid aspiration (19, 42), it is not understood how these two insults might synergistically drive the epithelial barrier disruption and airway neutrophil recruitment that characterize the early exudative phase of acute lung injury (ALI). We know from previous work that acid aspiration and inhaled LPS can independently lead to ALI, each exhibiting a modest disruption of lung function (1, 20). We hypothesized, however, that with the added disruption of barrier function at the time of acid aspiration, the combined exposure to acid and LPS would synergistically amplify the progression of lung injury and the disruption of lung function, primarily through an acid-mediated amplification in LPS signaling. We thus examined the separate and combined in vivo effects of acid aspiration and inhaled LPS on inflammatory cytokine signaling, cellular inflammation, epithelial barrier disruption, and lung mechanics in a mouse model of ALI. We then examined the in vitro response of cultured cells to air space fluid obtained from mice following acid aspiration, both in the presence and absence of LPS. We then examined the pH and burden of LPS within

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routinely obtained endotracheal and gastric fluid sampling from patients in the medical intensive care unit (MICU). Our findings support the notion of synergy between acid injury and LPS in the pathogenesis of lung injury in mice and open new potential explanations for the variable clinical course following aspiration in human patients. Some of the results of these studies have been previously reported in the form of an abstract (4, 38).

MATERIALS AND METHODS

Injury protocol. All protocols were approved following review by the Institutional Animal Care and Use Committee at the University of Vermont. Eight- to 10-wk-old female C57BL/6 mice (Jackson Laboratory) were used for all experimental protocols and were habituated and housed in a pathogen-free facility at the University of Vermont. Mice were deeply anesthetized in a Plexiglas chamber with 5% inhaled isoflurane until breathing rate slowed to one per second and then hung semirecumbently by their teeth, and their tongue was retracted with padded forceps to allow for visualization of the vocal cords. A volume of 4 μl/g of normal saline or HCl in saline, titrated to a pH of 1.3 (~0.02 M), was then slowly pipetted onto the vocal cords and aspirated by the mice during deep agonal breathing. Following full recovery from anesthesia, all mice were monitored and assigned 1 h later to receive either aerosolized lipopolysaccharide (LPS; *Escherichia Coli* 0111:B4, Sigma, St. Louis, MO; 3×10^6 EU/ml) over 15 min or nothing. Twenty-five hours following aspiration (24 h after LPS aerosol), FITC-dextran (2.5 mg/ml FD4; Sigma) was administered via tail vein injection (25 mg/kg; ~200 μl), followed by anesthesia with intraperitoneal pentobarbital (90 mg/kg) and connection to a *flexiVent* mechanical ventilator (SCIREQ, Montreal, Quebec, Canada) via surgical tracheostomy with an 18-gauge metal cannula. Mice received a tidal volume of 10 ml/kg at 180 breaths/min with positive end-expiratory pressure (PEEP) of 3 cmH_2O. The mice were paralyzed with intraperitoneally with pancuronium bromide (0.5 mg/kg), and heart rate was monitored by electrocardiogram to ensure deep anesthesia.

Following a 5-min stabilization period, the level of PEEP was set at 6 cmH_2O, and a 1.0-ml deep inflation (DI) was delivered twice over 4 s (constant flow, pressure limit of 30 cmH_2O). The mice were then returned to quasisinusoidal ventilation at 180 breaths/min. Respiratory impedance (*Zrs*) was determined via Fourier transform from the signals of ventilator piston volume displacement and cylinder pressure, both measured during 2-s oscillatory volume perturbations immediately following DI and then subsequently every 20 s for 8 min. The perturbations were composed of 13 superimposed sine waves with frequencies ranging between 1 and 20.5 Hz and mutually primed to reduce harmonic distortion (2, 3). *Zrs* itself was interpreted by being fit to the constant phase model of the viscoelastic lung, from which the parameters *G* and *H* were derived. The parameters *G* and *H*, respectively, characterize the dissipative (tissue damping) and elastic properties of the lung tissues (18). This same protocol was then repeated at a PEEP of 3 and 1 cmH_2O.

At the end of each post-DI *Zrs* measurement/tracking period, a quasistatic pressure-volume (PV) curve was obtained from resting residual lung volume by first dropping PEEP to 0 cmH_2O and then immediately delivering seven steps of inspiratory volume to a total volume of 40 ml/kg, followed by seven equal expiratory steps, pausing at each step for 1 s. Plateau cylinder pressure was measured during each pause and plotted against piston displacement volume; the latter was corrected for gas compression.

Bronchoalveolar lavage fluid collection and analysis. Following *Zrs* measurements, the mice were euthanized with pentobarbital (200 mg/kg ip), and bronchoalveolar lavage fluid (BALF) was obtained by instilling 1 ml of phosphate-buffered saline through the tracheal cannula and suctioning back for a return of ~0.9 ml. Immediately following collection, the BALF was centrifuged, and the supernatant was stored at −80°C. As an index of epithelial barrier integrity at 24 h, BALF samples were analyzed for FITC-dextran concentrations (administered before anesthesia) (2, 17) by loading 50 μl of BALF (in duplicate) onto a microwell plate, exciting the plate at 485 nm, and reading fluorescence at 528 nm. The concentration of FITC-dextran was then interpolated from a linear standard of fluorescence vs. graded concentrations of FITC-dextran.

To evaluate the time course of inflammatory change and barrier disruption independent of any potential effect from mechanical ventilation, additional mice ($n = 6$ per group and time point) were again anesthetized with isoflurane for aspiration of 4 μl/gm of normal saline or HCl in saline (pH 1.3) and assigned to receive aerosolized LPS or nothing 1 h later. The mice were euthanized at 2, 5, 13, 25, or 49 h following aspiration (1, 4, 12, 24, or 48 h following LPS), at which time BALF was collected in the same manner described above. The spun BALF cell pellets were resuspended and stained, and the total cell counts were determined manually using a hemacytometer. Cytospun slides were stained with hematoxylin and eosin for neutrophil differential count determination. The protein content of the BALF supernatant was calculated using a colorimetric assay (Bio-Rad, Hercules, CA), standardized to graded concentrations of bovine serum albumin. BALF supernatant specimens were analyzed in duplicate for their concentration of the inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, murine keratinocyte cytokine (KC), regulated on activation normal T cells expressed and secreted (RANTES), and granulocyte colony stimulating factor (GCSF) using a Bio-Plex murine multi-plex plate (Bio-Rad) and Bio-Plex 200 plate reader according to manufacture protocol.

For in vitro studies, additional mice ($n = 5$ per group) were anesthetized for acid or saline aspiration and collection of BALF 1 h later. Separate mice received an intraperitoneal injection of thioglycolate (1.5 ml, 3% sterile solution) and 4 days later were deeply anesthetized with isoflurane and euthanatized via cervical dislocation (to prevent peritoneal contamination). The abdominal skin was bluntly dissected, an 18-gauge needle was inserted into the abdomen, and RPMI medium slowly was injected and reaspirated to collect peritoneal cells. The cells were cultured in serum-containing media overnight. The next day, the cells were washed and plated in serum-free media, along with 200 μl cell-free BALF supernatant from either saline or acid-exposed mice, and 5 μl of LPS (50 ng) or sterile saline. The cells were then incubated at 37°C, and equal volumes of mixed media were drawn off the wells at 1, 2, 3, and 4 h following LPS stimulation and then assayed for TNF-α (BD OptEIA, Ontario, Canada) as a marker of LPS-induced cell activation. Additional mice ($n = 6$ per group) were anesthetized with isoflurane to aspirate 4 μl/g of normal saline or HCl in saline (pH 1.3) and euthanized either 1 or 5 h following aspiration for procurement of BALF. BALF levels of LPS binding protein (LBP) were quantified using commercial ELISA kits (Cell Sciences, Canton, MA).

Human sample acquisition and testing. We obtained approval from our local Institutional Review Board for a waiver of consent to collect routinely obtained and discarded gastric and tracheal aspirate fluid specimens from intubated and mechanically ventilated patients in the MICU within 24 h of tracheal intubation and gastric tube placement. Respiratory therapists and nurses were briefed on specimen collection technique, which included clearance to the inline tracheal suctioning catheter with 5 cc of sterile saline before specimen collection. Patients were defined as being "high risk" for aspiration (8 of 20) if they carried a diagnosis of aspiration pneumonia, encephalopathy, alcohol withdrawal, or intubated at the time of seizures or cardiopulmonary arrest (failure to protect airway). Patients were designated as having ARDS if they carried a diagnosis of ARDS, had bilateral air space edema, and had a $Pa_{O_2}:F_{I_{O_2}}$ ratio <300. Samples were kept in a sterile syringe, transferred to a sterile 15-ml conical tube, placed on ice, and immediately transferred to our laboratory for storage at −80°C. The pH of each specimen was later measured with a micro-pH meter and

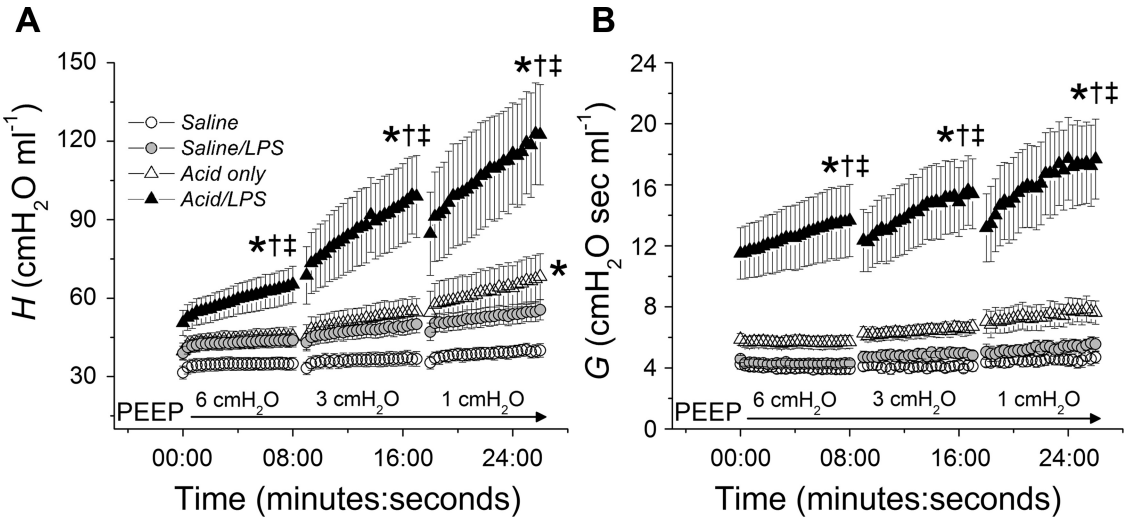

Fig. 1. *A*: progressive rise in mean elastance (*H*; ±SE bars) following deep inflation (DI) at positive end-expiratory pressure (PEEP) levels of 6, 3, and 1 cmH_2O for mice following saline aspiration only (open circles), acid aspiration only (open triangles), saline aspiration followed by LPS aerosol (grey circles), and acid aspiration followed by LPS aerosol (black triangles). *B*: progressive rises in mean tissue resistance (*G*; ±SE bars) following DI, derived from same impedance data as *A*. *$P < 0.05$, compared with *saline-only*; †$P < 0.05$, compared with *saline/LPS*; ‡$P < 0.05$, compared with *acid-only* group.

batched analyzed for LPS on a microwell plate using a limulus amoebocyte lysate chromogenic endpoint assay (LAL; Cell Sciences). Gastric samples were diluted with LPS-free water and loaded via LPS-free pipette tips into the test wells. Tracheal aspirate samples were diluted 1:1 with a 0.1% endotoxin-free solution of dithiothreitol to disrupt disulfide bonds within the mucin. LPS concentrations were determined from a serial dilution standard curve, which included LPS-free water as blank. Background LPS levels, measured in LPS-free water transferred into and out of the sample storage containers, was negligible.

Statistics. All graphing and statistical analyses were performed using Origin (version 8.1; Northampton, MA) and GraphPad PRISM (version 5.03; La Jolla, CA) software. Multiple measures ANOVA was used to detect a significant effect of injury type and PEEP on measures of impedance and PV hysteresis and to detect a significant effect of injury type and time on cell counts, BAL protein, and cytokine values. Provided a significant effect was demonstrated overall and a significant interaction was demonstrated between injury and PEEP or between injury and time ($P < 0.05$), each ANOVA was then followed by post hoc Bonferonni tests for means comparison between groups.

RESULTS

The aspiration of saline followed by LPS aerosol (*saline/LPS*) and the isolated aspiration of acid (*acid-only*) each led to roughly equivalent rises in *H* (ΔH) and *G* following DI (Fig. 1, *A* and *B*), and in PV hysteresis (Fig. 2) compared with saline aspiration only. This was despite the *saline/LPS* injury recruiting significantly more air space neutrophils than did *acid-only* aspiration (Fig. 3). However, the combination of acid aspiration and LPS aerosol (*acid/LPS*) led to significantly greater increases in baseline and post-DI rises in *H* and *G* and increases in PV hysteresis at every level of PEEP. Furthermore, these parameters were increased to an extent that exceeded the additive effects of the separate injuries alone (Fig. 1). This same degree of synergy between acid and LPS aerosol was seen in their combined effect on BALF neutrophil counts (Fig. 3) and on markers of barrier disruption, specifically BALF levels of protein and FITC-dextran (Fig. 4, *A* and *B*).

Figure 3 demonstrates that the recruitment of air space neutrophils follows a different time course depending on the type of injury, with BALF neutrophils peaking in the *acid-only* group at 13 h, but peaking at 24 h in the *saline/LPS* and *acid/LPS* groups. In a similar manner, Fig. 5 shows that in the setting of combined *acid/LPS* injury BALF cytokines peak at significantly higher levels than in any other group and the peaks temporally correspond with those of the *saline/LPS* group more than those of the *acid-only* group.

Figure 6 demonstrates that cultured peritoneal macrophages release TNF-α in response to LPS (Fig. 6, shaded symbols). In the presence of BALF supernatant from saline-exposed mice, cultured peritoneal macrophages release negligible levels of

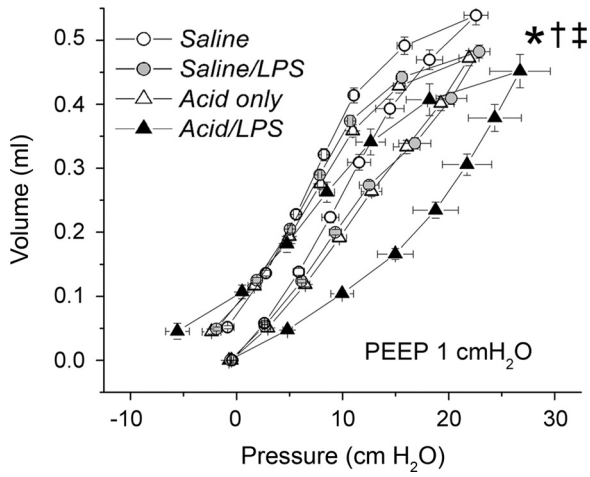

Fig. 2. Pressure-volume (PV) curves, derived from mean pressure and volume (±SE bars) obtained from 0 pressure, following ventilation at a PEEP levels of 1 cmH_2O in mice following saline aspiration only (open circles), acid aspiration only (open triangles), saline aspiration followed by LPS aerosol (grey circles), and acid aspiration followed by LPS aerosol (black triangles). The PV curve from mice following combined acid aspiration and LPS aerosol demonstrates increased hysteresis; *$P < 0.05$, compared with *saline-only*; †$P < 0.05$, compared with *saline/LPS*; ‡$P < 0.05$, compared with *acid-only* group.

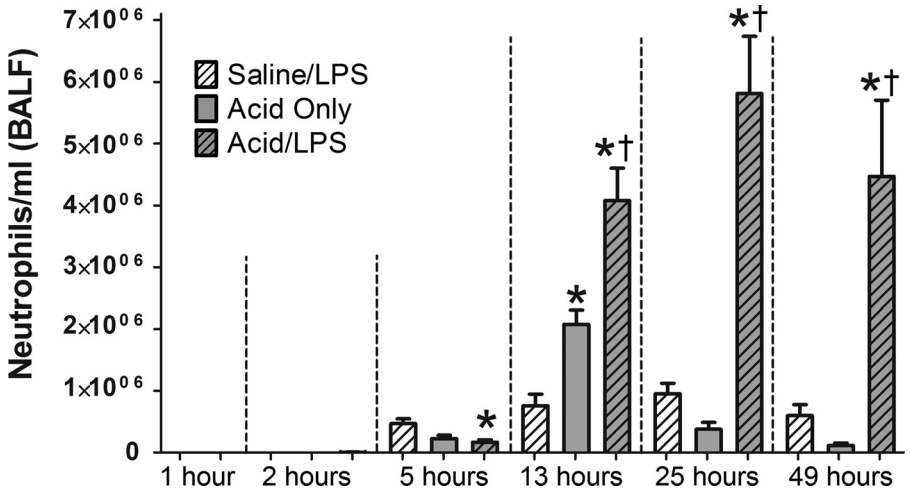

Fig. 3. Mean neutrophil counts (±SE bars) in bronchoalveolar lavage fluid (BALF) obtained at 1, 2, 5, 13, 25, and 49 h following aspiration in mice with saline aspiration only (open white bars, 1 h only), acid aspiration only (open grey bars), saline aspiration followed by LPS aerosol (white hatched bars), and acid aspiration followed by LPS aerosol (grey hatched bars). *$P < 0.05$, compared with *saline/LPS*; †$P < 0.05$, compared with *acid-only* group. Low neutrophil counts at 1 and 2 h not seen due to scale of *y*-axis.

TNF-α over 4 h in the absence of LPS, but the cells do release TNF-α when coincubated with LPS. In contrast, cells coincubated with pH-neutralized BALF supernatant from acid-exposed mice have a significantly greater basal release of TNF-α in the absence of LPS compared with cells exposed to BALF from saline-expose mice. Furthermore, the release of TNF-α from cultured macrophages in response to LPS was markedly amplified when coincubated with BALF from acid-exposed mice but significantly less so when coincubated with BALF from saline-exposed mice, particularly by 2 and 3 h (Fig. 6). Figure 7 demonstrates that BALF obtained from mice after acid aspiration has higher levels of LBP at 1 and 5 h following aspiration compared with BALF obtained from naïve mice (ANOVA, $P = 0.0043$) and mice enduring saline aspiration (ANOVA, $P = 0.0034$), but no significant difference existed between naïve and saline-exposed mice (ANOVA, $P = 0.93$).

For samples obtained from mechanically ventilated human subjects in the ICU, Fig. 8*A* demonstrates that there was a wide range of measurable LPS levels in gastric and tracheal aspirates, and a cluster of outliers with very high gastric LPS levels. Of these outliers with exceptionally high gastric LPS, the majority (5 out of 7) had tracheal aspirate LPS levels that fell within the upper measured range (between 40 and 600 U/ml), but because of the wide range of tracheal LPS levels in patients with low gastric levels, a direct correlation could not be demonstrated (Fig. 8*B*). Tracheal aspirate pH (data not shown) was higher on average and closer to normal physiologic pH among patients with ARDS than in those without ARDS (7.20 ± 0.14 vs. 6.95 ± 0.085), but the difference was not significant ($P = 0.13$). Mean gastric pH (not shown) was not significantly lower in patients with ARDS than those without (4.57 ± 1.03 vs. 6.10 ± 0.48; $P = 0.14$). Figure 8*C* demonstrates that mean tracheal LPS levels were higher among those at high risk of aspiration than those at normal risk ($P = 0.025$). When examining only those patients with moderately high levels of tracheal LPS (≥20 U/ml), gastric aspirates from patients with ARDS demonstrated a nonsignificant trend toward a lower mean pH than in patients without ARDS (3.17 ± 1.27 vs. 6.24 ± 0.64; $P = 0.051$; data not shown).

DISCUSSION

Our animal data demonstrate that LPS aerosol exposure and acid aspiration lead to modest and near equivalent disruptions in barrier function and lung mechanics when employed separately, but when delivered in staggered combination, they lead to a severe disruption of lung mechanics and a sustained and progressive inflammatory injury. Following separate exposures to LPS aerosol or acid aspiration, the recruitment of air space neutrophils progresses along different time courses. However, the timing of peaks in neutrophil counts observed in the combined *acid/LPS* group more closely corresponds with that

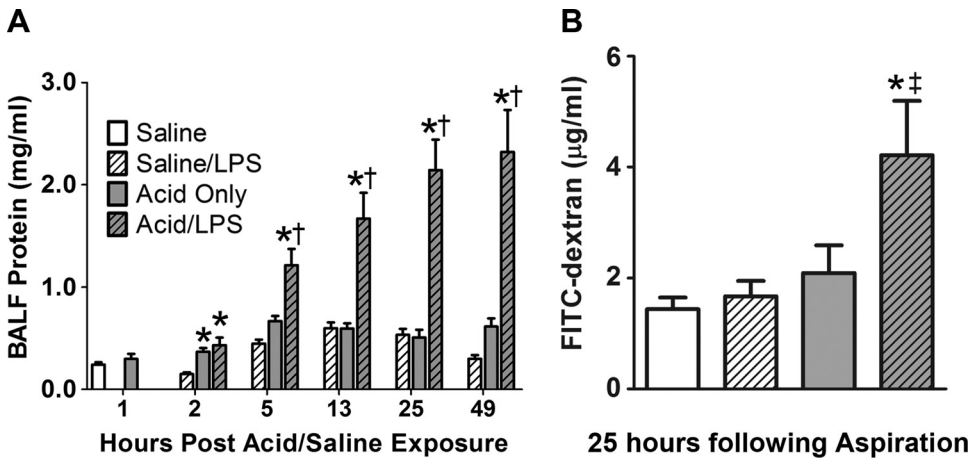

Fig. 4. *A*: mean protein levels (±SE bars) in BALF obtained at 1, 2, 5, 13, 25, and 49 h following aspiration in mice with saline aspiration only (open white bars, 1 h only), acid aspiration only (open grey bars), saline aspiration followed by LPS aerosol (white hatched bars), and acid aspiration followed by LPS aerosol (grey hatched bars). *$P < 0.05$, compared with saline/LPS; †$P < 0.05$, compared with *acid-only* group. *B*: mean FITC-dextran levels (±SE bars) in BALF obtained from mechanically ventilated mice 24 h following aspiration in similarly marked bars. *$P < 0.05$, compared with *saline/LPS*; ‡$P < 0.05$, compared with *saline-only* group.

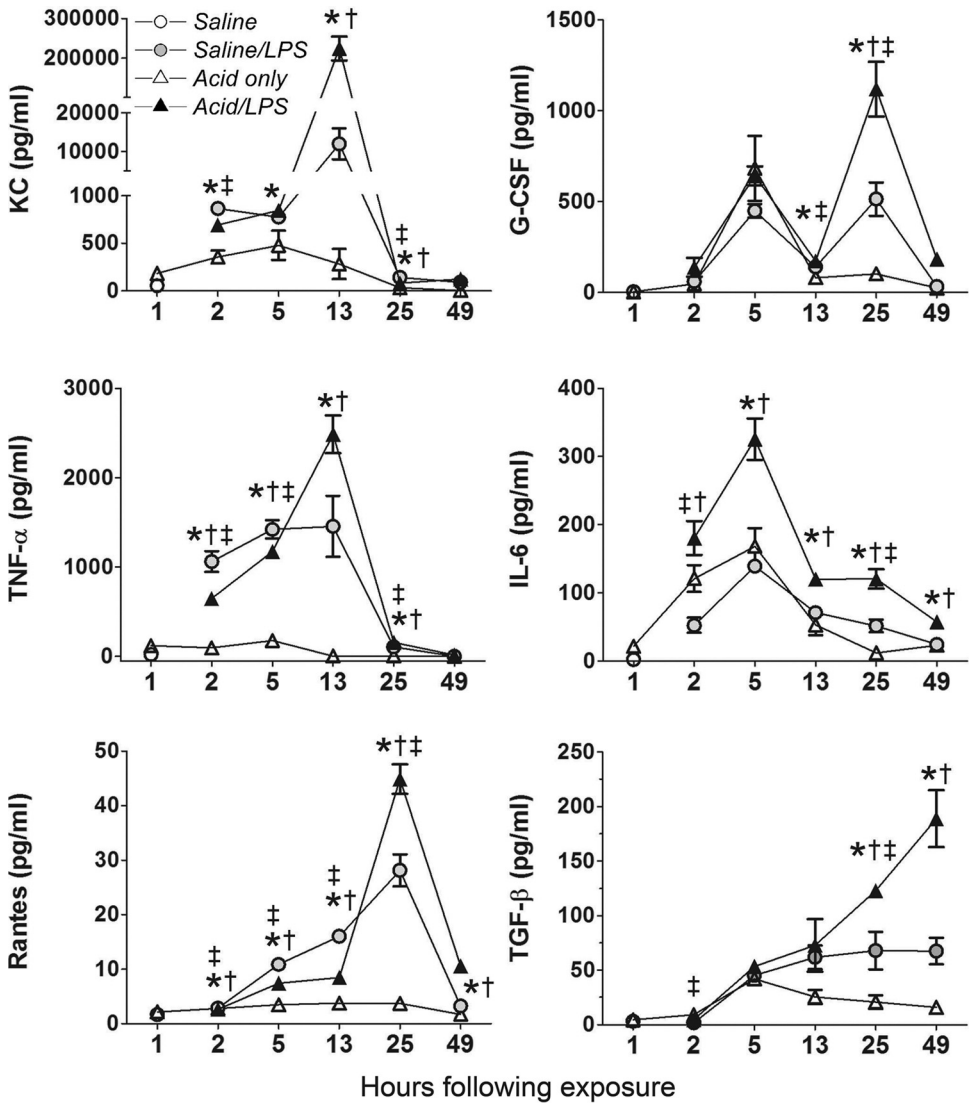

Fig. 5. Mean levels (±SE bars) of key proinflammatory cytokines in BALF obtained at 1, 2, 5, 13, 25, and 49 h following aspiration in mice following saline aspiration only (open circles, 1 h only), acid aspiration only (open triangles), saline aspiration followed by LPS aerosol (grey circles), and acid aspiration followed by LPS aerosol (black triangles). KC, keratinocyte cytokine; RANTES, regulated on activation normal T cells expressed and secreted; GCSF, granulocyte colony stimulating factor. *$P < 0.05$, significant difference comparing *acid/LPS* and *acid-only* groups; †$P < 0.05$, comparing *acid/LPS* and *saline/LPS* groups; ‡$P < 0.05$, comparing *saline/LPS* and *acid-only* groups.

of the *saline/LPS* group than with that of the *acid-only* group, suggesting that acid injury is augmenting LPS-mediated neutrophil recruitment, as opposed to the alternative order. To examine this notion more closely, we examined the time course of key air space neutrophil chemokines and growth factors under each injury protocol. Similar to the recruitment of air space neutrophils, the timing of peaks in BALF cytokine levels in the *acid/LPS* group more closely mimics that of the *saline/LPS* group than that of the *acid-only* group, particularly with respect to key neutrophil recruiting chemokines, such as murine KC and GCSF. When we further examined this issue at the in vitro level, we found that proinflammatory cascades (i.e., TNF-α release) can be triggered separately by the pH-neutralized BALF (in buffered saline) of acid-exposed mice or by LPS and that the same synergy exists between these stimuli when delivered in combination. The response of macrophages to cell-free, pH-neutralized BALF supernatant, and the associated synergy with LPS, strongly suggest that the downstream inflammatory consequences of combined acid aspiration and LPS exposure are not necessarily due to the direct pH effects of acid on inflammatory cells themselves. Our results rather suggest that the synergy observed in our model is due to an initial low pH-mediated release (possibly from direct injury of the epithelium) of some soluble mediator into the alveolar lining fluid that can amplify LPS signaling at the point of injury. In fact, a low pH environment, if anything, appears to have a downregulatory effect on alveolar macrophage function and cytokine release (12), lending greater support to our theory that the synergy imparted by acid in our in vivo model is due to a direct caustic injury to the epithelium and barrier function.

With investigation into the potential mechanisms for this amplified LPS signaling following acid aspiration, we found that air space levels of the key LPS signaling molecule LBP were significantly higher 1 h following acid aspiration compared with saline aspiration and that this elevation is sustained after 5 h (Fig. 7). It remains unclear from these findings whether LBP levels were elevated as a result of increased epithelial expression and elaboration of LBP (9) or simply due to increased leakage from plasma into the air space. The latter case seems more likely given the observed exudation of protein and FITC-dextran into the air space following acid aspiration (Fig. 4), combined with the fact that far greater basal levels of

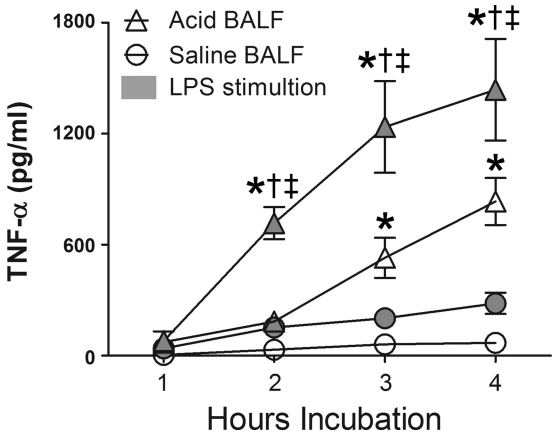

Fig. 6. Mean TNF-α levels (±SE bars) measured in media retrieved at 1, 2, 3, and 4 h from cultured peritoneal macrophages coincubated with lavage fluid of mice following acid aspiration (open triangles) or saline aspiration (open circles), either in the presence of sterile saline (white fill) or 50 ng of LPS (grey fill). *$P < 0.05$, compared with saline lavage fluid and no LPS; †$P < 0.05$, compared with saline lavage fluid with LPS; ‡$P < 0.05$, compared with acid aspiration lavage fluid without LPS.

LBP are found in the circulating serum (μg/ml) than in the alveolar compartment (ng/ml) (7, 11).

Although the present study is the first to report on the colocalized effects of acid aspiration and endotoxin on lung injury, we are not the first to examine the interaction between acid and endotoxin in the lung. Yamada et al. (42) examined a much different dual compartmental exposure in rats using combined delivery, but they separately delivered acid and endotoxin to the left and right lung, respectively, and separated exposure by 24 h. This injury yielded increased myeloperoxidase activity, barrier disruption, and TNF-α release, more so in the acid-exposed lung than in the LPS-exposed lung but not with the same degree of synergy observed in the present study. The authors interpreted this as evidence for acid "priming" the lung to subsequent endotoxin exposure.

Despite using a much weaker concentration of acid than the prior study by Yamada et al., our model was able to demonstrate a much greater synergy between acid and LPS, likely stemming from a number of possible factors. For one, our injury model is one of more colocalized exposure, both anatomically and temporally, exposing both lungs and allowing only 1-h time passage between exposures. Secondly, the previous study followed the injury out only 4 h from the last exposure. We also examined our model at 4 h from exposure but found that the synergy was much greater at 24 and 48 h following exposure. Given that we (see Fig. 6*A*) and others have shown that air space TNF-α levels rise within the first 24 h of acid aspiration (29, 30, 32), it could be that acid exposure "primes" the lung to subsequent LPS exposure via TNF-α (16, 27) when exposures are separated by a longer interval of time. In our model, because TNF-α levels in BALF 1 h following acid aspiration were significantly elevated above those of saline aspiration controls (119.93 ± 30.1 vs. 21.4 ± 3.4 pg/ml), we cannot entirely exclude this potential explanation for the synergy observed in our model (Fig. 5). Certainly other investigators have demonstrated TNF-α signaling to be crucial to the pathogenesis of acid aspiration lung injury (8). However, given that TNF-α levels in media from cultured macrophages were not significantly different between wells 1 h after coincubation with BALF from saline or acid-exposed mice (Fig. 6), we believe TNF-α priming is unlikely to be playing a significant role in the synergy demonstrated in our in vitro model.

The importance of our findings is underscored by the fact that while aspiration of gastric contents is arguably one of the most widely recognized causes of ALI (10, 13, 39), it remains unclear why the clinical course following gastric aspiration is so unpredictable. It is notable that for yet unclear reasons some patient will develop a self-limited pneumonitis following aspiration while others will progress to ARDS and death (22, 41). Yet certainly there are conceivable clinical scenarios in which the combined and colocalized exposure of the lungs to both acid and endotoxin can occur among hospitalized patients. Not surprisingly, air space and circulating endotoxin in the form of LPS are typically elevated in the setting of pneumonia or sepsis from Gram-negative infections (23, 28). However, LPS is an endotoxin so ubiquitous in our environment that small amounts can be routinely detected within the lung and plasma of mechanically ventilated and critically ill patients without any documented pneumonia or sepsis (23, 28). Data from the tracheal aspirates of our own ICU subjects corroborate these findings, with measureable levels of LPS in all of our samples, both in those at high and normal risk of aspiration (Fig. 8*A*). One potential source of this endotoxin, bacterial colonization of the stomach, has been widely demonstrated in mechanically ventilated patients (6, 14, 34), and gastric aspirates from our own human subjects demonstrate a wide range of gastric LPS burden in mechanically ventilated ICU patients (Fig. 8*A*) Furthermore, aspiration of acidic gastric contents into the airways has been shown to occur within hours of tracheal intubation (33, 35). It is thus highly plausible that LPS could sporadically serve as an adjuvant inflammatory insult in the setting of aspiration-pneumonitis, promoting the progression of a typically self-limited injury to one of fulminate ARDS. When examining samples from those subjects with ARDS in our cohort, it would appear that the pH of endotracheal aspirate is of less clinical relevance than endotracheal LPS burden. In fact, endotracheal aspirate pH of patients with ARDS was actually slightly higher and closer to physiologic pH (7.4) compared with non-ARDS patients (data not shown). This may be due to a greater buffering capacity from increased volumes

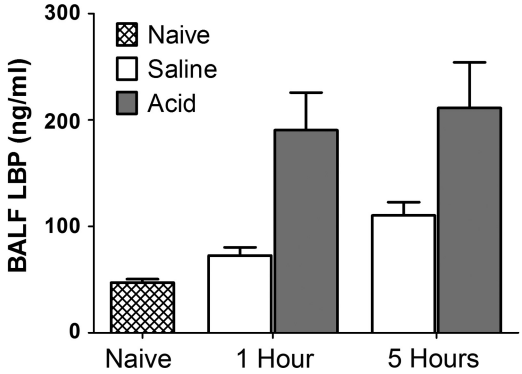

Fig. 7. Mean LPS-binding protein (LBP) levels (±SE bars) measured in bronchoalveolar lavage fluid obtained from naïve mice (white hashed bar) and mice 1 or 5 h following saline (white bars) or acid (grey bars) aspiration. *$P < 0.05$ by ANOVA, compared with naïve mice. †$P < 0.05$ by ANOVA, compared with saline aspiration.

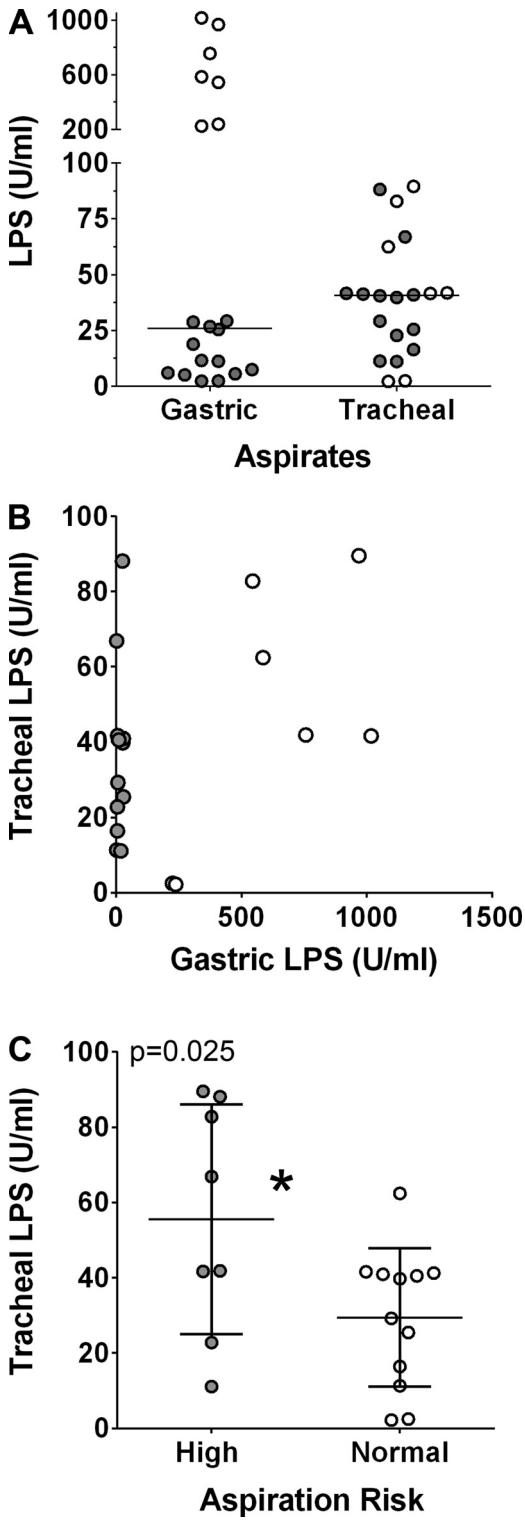

Fig. 8. Dot plot of human subject measurements of gastric and tracheal LPS (U/ml; *A*) and identity plot of human subject samples, plotting endotracheal LPS against gastric LPS levels (*B*). White (unfilled) circles identify a cluster of outliers with geometrically higher gastric LPS levels, also identified and matched to their corresponding endotracheal aspirate levels (*A*, *right*), and separately plotted in *B*. *C*: dot plots of human subject measurements of endotracheal LPS (U/ml) levels in patients at high and normal risk of aspiration, with higher mean airway LPS levels in those at higher risk of aspiration (*$P = 0.025$).

of plasma-rich edema fluid in the airways of patients with ARDS having impaired alveolar fluid clearance (40), but this is purely speculative. Nevertheless, our data do demonstrate a higher endotracheal aspirate LPS burden in patients at high risk for aspiration (Fig. 8*C*). When focusing only on those patients with a high airway LPS burden (a potential "single insult"), those with ARDS tended to have a lower gastric pH (a potential "second insult"), lending some credence to our two-hit animal model of lung injury. Unfortunately, the limited number of subjects falling into this category of high airway LPS with ARDS (3 patients) did not drive a statistically significant difference.

Although our clinical data are compelling and seem to support the clinical relevance our two-hit model, we must acknowledge that the limited sample number, weak statistical power, and poor correlation between gastric and airway LPS perhaps generate more questions than answers. Furthermore, the assignment of patients having a perceived "increase risk of aspiration" is open to bias. There is also the unfortunate barrier of impracticality when considering a follow-up analysis of gastric and airway pH and LPS before and after aspiration that can neither be anticipated or even entirely confirmed. It is also likely that gastric pH at the time we collected samples was artificially elevated by the use of pharmacologic gastric ulcer prophylaxis in all but two of our subjects (just intubated at time of collection). This current standard of care is likely to limit the clinical utility of airway or gastric pH measurement in the ICU. However, our results suggest that the utility and prognostic value of measuring airway and gut LPS burden might deserve further investigation.

Conclusions. Our study provides a compelling argument for some patients developing severe ARDS after coexposure to acid and LPS at or surrounding the time of aspiration. Variations in the acidity of gastric contents (15, 37) and the sporadic presence of air space LPS surrounding the time of aspiration (23) could help explain the variable clinical course of patients following witnessed or suspected aspiration of gastric contents (22, 41). The present study demonstrates that synergy exists in our animal model between these two insults. Human subject data demonstrating variable levels of airway LPS and a suggestion of higher airway LPS burden in those patients at highest risk for aspiration provide compelling support for the relevance of our animal model findings. Furthermore, the in vitro response of cultured cells to LPS and BALF from acid-injured mice suggests that this synergy arises from an acid-injury-driven amplification of LPS signaling, imparted by some soluble mediator(s) in the airway/alveolar lining fluid. Our data demonstrate that following acid aspiration the pH-buffered alveolar lining fluid contains one or more mediators capable of amplifying the inflammatory response to LPS, yet this fluid is also capable of stimulating inflammatory signaling on its own (in the absence of LPS) and is not pH dependent, the pH likely having been normalized by the normal buffering capacity of alveolar lining fluid long before this step. Thus acid aspiration leads to the elaboration of a single (or several) soluble mediator(s) that both "prime" macrophages to subsequent LPS exposure but also drive a separate inflammatory injury independent of LPS. Identifying which mediators are responsible for either or both phenomena could 1 day perhaps help clinicians to preemptively interrupt the progression of aspiration pneumonitis to ARDS.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

K.T., M.N., J.A.v.R., J.L.A., and J.C. performed experiments; K.T., J.A.v.R., and G.B.A. analyzed data; K.T. and G.B.A. prepared figures; G.B.A. conception and design of research; G.B.A. interpreted results of experiments; G.B.A. drafted manuscript; G.B.A. edited and revised manuscript; G.B.A. approved final version of manuscript.

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