Microarray analysis of regional cellular responses to local mechanical stress in acute lung injury

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IN RECENT YEARS, THE FOCUS of research into acute lung injury (ALI) has shifted from primarily mechanical and supportive management towards investigation into the cellular and molecular basis of the disease process. However, despite a great deal of data suggesting interactions between mechanical stress, inflammation, and the development of lung injury, the pathogenesis of ventilator-associated lung injury (VALI) is not well understood. A hallmark of human ALI that is not captured in many small animal models is the marked heterogeneity of tissue involvement (19). Mechanical and biological phenomena potentially contributing to VALI vary widely throughout the lung and may be highly dependent on particular supportive interventions. Recognizing this, management strategies have implicitly sought to reduce this heterogeneity so as to minimize the presumed “injurious” mechanical events (such as overdistension or air space opening/closing) while maintaining adequate gas exchange for life support (21).

We believe that VALI does in fact have its origin in inflammatory and other cellular responses in lung tissue exposed to (and possibly, predisposed to injury from) mechanical stress and hypothesize that these responses will vary throughout the heterogeneous injured lung in relation to local mechanical events. To explore this hypothesis, we assessed regional cellular responses by genomic microarray analysis, correlating changes in gene expression with the specific regional mechanical stresses imposed on that local tissue, in a novel canine model of unilateral lung injury. With each animal serving as its own control, the microarray-based genomic approaches (cross-species hybridization to the Affymetrix Human U133A GeneChip) provide broad characterization of regional cellular responses which, combined with functional computed tomography (CT) imaging for the noninvasive measurement of regional mechanical stress, makes possible the investigation of in vivo local cellular responses in the lung associated with heterogeneous VALI. Our goal was to establish whether regional cellular responses to local conditions could be determined and provide a novel window into understanding the mechanism of development of VALI.

EXPERIMENTAL PROCEDURES

Animal Preparation and Experimental Protocol

All experimental procedures were approved by the Johns Hopkins University Animal Care and Use Committee. Three mongrel dogs (weight 21.3 ± 1.5 kg) were anesthetized with 25 mg/kg pentobarbital intravenously and instrumented with femoral arterial and venous catheters. Anesthesia was maintained with additional pentobarbital (5 mg/kg iv every hour and when indicated), and muscle relaxation was provided by pancuronium (3 mg bolus and 0.5 mg hourly iv). A 39 or 41 French double-lumen endobronchial tube (Mallinkrodt, St. Louis, MO) was placed via a tracheostomy, and position was confirmed by fiber-optic bronchoscopy. The animals were ventilated with a “dual...
piston” large animal ventilator (Harvard Apparatus, Holliston, MA) permitting independent control of tidal volume (Vt), inspired oxygen fraction (FIO2 1.0), positive end-expiratory pressure (PEEP), measurement of airway opening pressure (Paw), and end-tidal partial pressure of carbon dioxide (ETPCO2) for each lung. Oxygen saturation (SO2) was continuously measured using a pulse oximeter applied to the tongue or ear, and ETPCO2, arterial blood pressure (P, Paw, and esophageal pressure (Pes) were continuously recorded. An infusion of lactated ringers (LR, 5–10 ml/kg h–1) was given for maintenance fluid replacement. Rectal or pulmonary artery temperature was maintained at 36 ± 1°C with radiant heat lamps. At the conclusion of the study, the animals were killed by exsanguination after supplemental pentobarbital (10 mg/kg iv).

After instrumentation, the individual lung Vt were adjusted to provide an ETPCO2 of 30–35 mmHg for each lung at a respiratory rate of 20 breaths/min. This procedure resulted in individual lung Vt (means ± SD) of 8.7 ± 0.3 ml/kg for the right lung and 6.1 ± 0.4 ml/kg for the left, approximating the normal distribution of lung volume. The left lung was then mildly injured by repeated lavage with warmed saline, 20 ml/kg repeated four times while switching the animal’s position between right and left lateral decubitus, with gravity drainage. For comparison, typical lung lavage injury protocols use 40–60 ml/kg (both lungs) for six to eight washes to achieve an arterial drainage. For comparison, typical lung lavage injury protocols use animal’s position between right and left lateral decubitus, with gravity drainage. For comparison, typical lung lavage injury protocols use 40–60 ml/kg (both lungs) for six to eight washes to achieve an arterial drainage.

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RESULTS

Unilateral Canine Lung Injury Model

Over the course of the 5-h observation period, the peak inspiratory pressure of the injured lungs approximately doubled, whereas that of the control lungs remained unchanged. \( \text{SaO}_2 \) remained at 99–100% at all times despite unilateral lung injury because of the use of a high \( \text{FiO}_2 \), and the presence of the control lung. All animals remained normotensive throughout the study without intervention. Lung wet/dry weight ratios (w/d, means ± SD) were lower in control lungs (5.76 ± 1.01) compared with injured lungs (8.08 ± 1.86, \( P < 0.01 \)), with w/d in injured lungs greater in base regions (9.14 ± 1.37) than apex regions (7.01 ± 1.70, \( P = 0.02 \)). There was no significant difference in w/d between base and apex regions in the control lungs (6.15 ± 1.20 vs. 5.38 ± 0.66, \( P = 0.13 \)).

Representative tissue histological sections show severe acute inflammation in the left (injured) lung base, with reduced inflammation in the left nondependent base (Fig. 1, A and B). In comparison, the right (control) lung sections revealed minimal inflammatory changes (Fig. 1, C and D). Apical sections of both control and injured lungs showed minimal inflammation.

CT imaging in one animal revealed increased lung density throughout the lavaged left lung, with preservation of normal aeration in the control right lung with the exception of mild dependent atelectasis (Fig. 2A). Apex-to-base profiles of average lung density from the cross-sectional images for the control lung showed a normal distribution of density for a supine dog (36), ranging from 80% air content at the apex to 50–60% toward the lung base. In contrast, the injured lung exhibited severe loss of aeration throughout except for regions toward the apex (Fig. 2D). Examining vertical gradients of lung density in subvolumes from the midapex and midbase regions showed steep aeration gradients in both injured and control lungs. At the apex, both lungs were well aerated in nondependent regions, but with the injured lung losing aeration much more rapidly toward the dependent regions. At the base, the injured lung was poorly aerated throughout (Fig. 2B). Voxel density histograms from dependent and nondependent regions of both lungs from the apex and base further illustrate the mechanical heterogeneity of these lung regions (Fig. 2C). This distribution of lung density, with greatest loss of aeration in dependent base regions, is consistent with patterns seen in humans with ALI/acute respiratory distress syndrome (ARDS) (45) as well as with the pattern of long-term injury seen in the lungs of ARDS survivors (18).

Regional Microarray Analysis

A total of 24 samples from three animals were successfully hybridized to 24 Affymetrix HG-U133A microarray chips and are included in this analysis. Since samples were taken from corresponding regions of the injured and control lungs of each animal, results from each regional injury sample were normalized to the within-animal control, a unique feature of this unilateral injury model. Initial analysis of expression profiles generated using Affymetrix MicroArray Suite (MAS 5.0) showed that ~14% (~3,000 of >22,000 probe sets analyzing 18,000 transcripts and variants) of the canine genome hybridized to corresponding human probe sets with an efficiency similar to human mRNA. Sequence comparison showed that this fraction of canine mRNAs represented canine genes that were highly homologous to their human counterparts. Overall
transcript detection by the cross-species hybridization of canine mRNA to the HG-U133A chip was much less sensitive (14%) than hybridization of human mRNA (typically 48%). However, application of canine-specific masking techniques (22, 23) in which nonhomologous probe sets between species are excluded from the usual multiprobe set matching requirements increased transcript detection to >65%.

Comparing regions, there were 308 and 595 genes significantly changed [defined as FDR <2% and average regional injured/control fold-change (FC) >1.7 or <-1.7] in the apex and base, respectively, of which 213 (69.2%) increased expression in the apex and 238 (40.0%) in the base; 367 genes were significantly differentially expressed between the apex and base (FDR <2% for apex/base difference and either apex and/or base injured/control FC >1.7 or <-1.7). Cluster analysis of significantly changed genes revealed groupings of differential regional gene expression changes, along with the most represented gene ontologies (12) (Fig. 3).

Regional gene expression differences were even more marked between dependent and nondependent samples from the lung base. Of the 1,438 genes with changed expression (FDR <2% and injured/control FC >1.7 or <-1.7) in the dependent samples, 55.1% had increased expression relative to control compared with 45.5% of the 1,221 changed genes in the nondependent samples. Expression was significantly different between the dependent and nondependent base regions in 1,544 genes and changed in opposite directions (FC >1.7 and <-1.7 in the 2 regions) in 63 genes (Table 1). These differences were not due to regional variation in the control samples, as there were no significant dependent/nondependent differences in control lung gene expression and only 18 apex/base differences, none of which appeared in any of the results described here. Cluster analysis of genes significantly changed between apex and base, along with their gene ontologies, are presented in Fig. 4.

The gene ontologies (12) most significantly changed between dependent and nondependent base samples are presented in Table 2. We identified 226 genes in the literature related to ALI (3, 10, 13, 22, 24, 29, 35, 60) or modified by cyclic stretch in an alveolar epithelial cell model (13) and compared them to the regional expression results. From this list of ALI candidate genes (including closely related genes from the same families), 87 genes exhibited differential gene expression between dependent and nondependent regions within the same lung lobe.
in these animals. A selection of 50 of these ALI-associated genes, along with additional highly changed genes (FDR < 2% and FC < -2.5 or >2.5) in our model, presented in Fig. 5, represents an extended list of cellular processes altered by the regional environment (see Supplemental Table 1 for the complete referenced list of these 50 differentially changed ALI-related or highly changed genes and Supplemental Table 2 for the complete list of 294 ALI-related or highly changed genes online at the AJP-Lung Cellular and Molecular Physiology web site.). In addition, MIAME compliant microarray data have been deposited at http://www.ncbi.nlm.nih.gov/geo under accession no. GSE1935.

### Validation Studies

GeneChip results for five different upregulated genes were validated using semiquantitative RT-PCR, showing good agreement in fold-change injured/control between samples taken from apex (n = 6) and base (n = 6) regions (Fig. 6). There were no statistically significant differences between microarray and RT-PCR measured fold-changes by paired t-test (P range 0.39 – 0.57).

### DISCUSSION

Ventilator management has been associated with patient outcome in ALI since the original description of ARDS by
Ashbaugh and colleagues in 1967 (5). Since that time, the advent of imaging studies using CT revealed the heterogeneous mechanical nature of ARDS (19, 20) and provided insight into the impact of ventilator management on regional mechanical events. Meanwhile, basic research in rodent models identified a relationship between presumed mechanical events, such as end-expiratory airways opening/closing and end-inspiratory overdistension, and the release of inflammatory cytokines (8, 44, 51) that could potentially account for the observation that most patients with ARDS die of multiorgan system failure (39), and further, that subjects with sepsis, trauma, and systemic syndromes appear susceptible to developing VALI (1, 33). The association between ALI, ventilator management, outcome, and inflammation has been strengthened in many subsequent basic and clinical studies (7, 25, 49, 52), including several that utilized genomic microarrays to explore broad mechanisms of cellular activation in ALI (3, 10, 13, 22, 35). Several physiological systems have been identified as important in the evolution of VALI, including coagulation, surfactant, stress response, cytoskeletal structure, oxidative injury, and endothelial/epithelial barrier dysfunction (2, 10, 16, 17, 22, 47). Despite these advances, understanding of the relationship between specific mechanical events in the lung and the cellular responses that result in the development or progression of VALI remains incomplete.

Our goal was to establish whether regional cellular responses to local mechanical conditions could be determined as a first step towards using these regional events as a novel window into understanding the mechanism of development of VALI. We developed a unique canine model of unilateral lung injury and mechanical ventilation to allow the comparison of control and injured tissue samples from the same animal. We chose the canine saline lavage model of ALI because the insult is primarily mechanical and serves to amplify the effects of mechanical ventilation by removing surfactant and reducing stability of peripheral air spaces, although a secondary inflammatory response does ensue (48). The progressive increase in peak inspiratory pressure over the 5 h seen only in the lavage lung indicates the interaction between mechanical ventilation and the initial lavage insult that is characteristic of ventilator-associated lung injury. Previous experience with this model had demonstrated significant mechanical heterogeneity (14, 34) not unlike that observed in patients with ARDS (18, 20), with dependent, basal flooding and air space collapse, intermediate zones of apparent air space opening and closing, nondependent overdistension, and relatively preserved apical regions, behavior confirmed by our CT data from one animal. Furthermore, successful cross-species hybridization to human microarrays had been described (38, 40, 57) and canine-specific microarrays were under development (4, 6, 26, 50).
Table 2. Ontology of altered gene expression between dependent and nondependent base regions

<table>
<thead>
<tr>
<th>GO ID</th>
<th>GO Name</th>
<th>GO Type</th>
<th>No. of Genes with FC &gt;1.7 or &lt;−1.7</th>
<th>No. of Measured Genes</th>
<th>No. of Genes in GO</th>
<th>Percent Genes Changed</th>
<th>Percent Genes Present</th>
<th>Z Score</th>
<th>Permuted P Value</th>
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<tr>
<td>8009</td>
<td>Chemokine activity</td>
<td>F</td>
<td>5</td>
<td>42</td>
<td>49</td>
<td>11.9</td>
<td>85.7</td>
<td>11.13</td>
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<tr>
<td>6954</td>
<td>Inflammatory response</td>
<td>P</td>
<td>6</td>
<td>153</td>
<td>171</td>
<td>3.9</td>
<td>89.5</td>
<td>7.14</td>
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<tr>
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<td>Immune response</td>
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<td>5</td>
<td>245</td>
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<td>79.8</td>
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<td>6.72</td>
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<td>8283</td>
<td>Cell proliferation</td>
<td>P</td>
<td>4</td>
<td>215</td>
<td>229</td>
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<td>93.9</td>
<td>5.91</td>
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<tr>
<td>5576</td>
<td>Extracellular region</td>
<td>C</td>
<td>7</td>
<td>391</td>
<td>504</td>
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<td>77.6</td>
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<td>Heparin binding</td>
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<td>3</td>
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<td>58</td>
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<td>Positive regulation of cell proliferation</td>
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<td>4</td>
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<td>2.3</td>
<td>81.1</td>
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Taken from genes with FDP <2 for dependent vs. nondependent expression and dependent and/or nondependent FC >1.7 or <−1.7 presented in Fig. 5. GOs with Z >1.96 and first node (no. of changed genes) >2 were considered significantly affected by ventilator-associated injury. GO, gene ontology; FC, fold-change injured/control; P, biological processes; F, molecular function; C, cellular component.

Finally, the canine model is of sufficient size to allow lung isolation and independent treatment, permitting the use of each subject as its own control to reduce the baseline variation inherent in unblinded subjects. The benefit of decreased intersubject variation from this control scheme should offset possible changes to the control lung from systemic “spillover” of inflammatory or other mediators from the injured lung (49, 51), although the possibility remains of false negative results from this effect.

Although it is known that there is regional mechanical heterogeneity within the injured lung (19), and we documented regional differences in tissue aeration, w/d ratio, and inflammation, there are potentially other factors that could influence these results. In addition to differences in regional ventilation, there are likely differences in regional perfusion within the injured lung. In the lavaged lung, dependent, poorly aerated regions reduce their perfusion via the homeostatic hypoxic pulmonary vasoconstriction response (15). Similarly, although postural repositioning was used in an attempt to uniformly distribute the saline lavage, the removal of surfactant and distribution of residual fluid may be nonuniform. Finally, the number and type of migratory inflammatory cells differ between regions. Thus the stimulus for the differential regional responses found may not be purely the local mechanical stresses but could reflect contributions of other heterogeneous aspects of this large animal injury model.

The extension of microarray technology to large animal models of disease has been limited by the lack of availability of species-specific gene chips, and thus cross-species hybridization has been increasingly utilized for this purpose (38, 40, 57). This approach is useful for gene products with an adequate degree of homology to their human counterparts, whereas failure to detect an expressed gene cannot distinguish between lack of stimulus effect vs. failure to hybridize because of species-specific sequence differences. The present call rate can be increased using masking techniques that either eliminate poorly functioning probe sets or relax the stringent criteria for detecting all the probe set sequences in Affymetrix-type arrays, since differences between species may be limited to only portions of the sequence (22, 23, 26). As our purpose was to demonstrate regional differences in gene expression in response to different mechanical stresses, false negatives are irrelevant with the acknowledgment that these results are but a subset of the gene expression changes that have occurred. Recent announcement of the availability of a commercial canine microarray from Affymetrix as well as the development of custom canine cDNA arrays (4, 6, 50) will facilitate more specific and exhaustive analysis in the future. In any event, the use of microarray data in this manner should be considered exploratory and any specific mechanistic inferences should be validated with more precise techniques.

Analysis of the microarray data for the injured vs. control lung showed significantly changed expression in 472 genes, of which 46% were increased. The functional groupings (ontologies) of these genes were similar to those we have previously described (22, 24), including angiogenesis, cell cycle arrest, inflammation, coagulation, and immune response. Confirming our initial hypothesis, there were large numbers of genes differentially regulated between the apex and base (Fig. 3) and between dependent and nondependent regions within the base (Table 1 and Figs. 4 and 5). These lung regions experience strikingly different mechanical environments, as evidenced by their CT density distributions (Fig. 2). Furthermore, imaging studies of ARDS survivors have shown that chronic lung damage, mostly bullous lesions, occurs predominantly in dependent and basal lung regions, whereas apical regions are relatively spared (18). The mechanical events related to artificial ventilation most commonly invoked as injurious to the lung are overdistension, airways repeatedly opening and closing with each breath, and to a lesser extent tissue collapse.
and/or flooding, although the data supporting these relationships in patients remain controversial (28). In our model, which used no PEEP in the injured lung, there was minimal evidence of overdistension based on the end-inspiratory criterion of 90% aeration by CT (54), and any overdistension present occurred toward the apex. Of course, it is also possible that differences in other regional properties, such as perfusion or migratory inflammatory cells, influenced the observed changes in gene expression.

Examination of the highly differentially regulated genes between apex and base regions reveals several genes commonly associated with ALI (vascular endothelial growth factor C, THBS1, transforming growth factor-β) as well as novel genes not previously described in the ALI literature. For example, the gene encoding the proposed proinflammatory cytokine Pre-B cell colony enhancing factor (PBEF) was one of the most upregulated genes in the injured lung. PBEF was originally described for its role in the maturation of B cell precursors (42) and was subsequently found to be upregulated in amniotic membranes from patients undergoing premature labor (especially with amniotic infections) (37). Epithelial cells from amniotic membranes also upregulate PBEF expression and/or flooding, although the data supporting these relationships in patients remain controversial (28). In our model, which used no PEEP in the injured lung, there was minimal evidence of overdistension based on the end-inspiratory criterion of >90% aeration by CT (54), and any overdistension present occurred toward the apex. Of course, it is also possible that differences in other regional properties, such as perfusion or migratory inflammatory cells, influenced the observed changes in gene expression.

Examination of the highly differentially regulated genes between apex and base regions reveals several genes commonly associated with ALI (vascular endothelial growth factor C, THBS1, transforming growth factor-β) as well as novel genes not previously described in the ALI literature. For example, the gene encoding the proposed proinflammatory cytokine Pre-B cell colony enhancing factor (PBEF) was one of the most upregulated genes in the injured lung. PBEF was originally described for its role in the maturation of B cell precursors (42) and was subsequently found to be upregulated in amniotic membranes from patients undergoing premature labor (especially with amniotic infections) (37). Epithelial cells from amniotic membranes also upregulate PBEF expression
when subjected to stretching in vitro (41), and human fetal membrane explants exposed to PBEF produce inflammatory cytokines and chemokines (43). Furthermore, PBEF was recently identified as a cell cycle regulator, delaying neutrophil apoptosis in experimental inflammation and clinical sepsis (32) and thus functions as a regulator of the inflammatory response. Thus PBEF represents a proinflammatory cytokine, with proposed roles in immune regulation and oxidative metabolism and known association to infected or stretched epithelial cells, which has now been identified as significantly upregulated in a primarily mechanical model of VALI. These observations led us to pursue in-depth investigation of its role in other ALI disease models and in patients with sepsis-related ALI, revealing a consistent upregulation of PBEF in murine and canine LPS-induced ALI, in cytokine and cyclic stretch lung endothelial cells, and increased PBEF protein in both bronchoalveolar lavage and serum of patients with ALI (60). Furthermore, analysis of two single nucleotide polymorphisms in patients with sepsis-associated ALI suggests that a susceptible haplotype (GC) in the PBEF promoter region is associated with a 7.7-fold higher risk of developing ALI (60), strongly supporting a role for PBEF both in the pathogenesis of ALI and as a biomarker for ALI susceptibility.

Among the genes differentially regulated within the lung base were several stress response proteins, notably the inducible 70-kDa heat shock protein (HSP) whose expression has been associated with protection from development of ALI (55). Three HSP family genes, along with related chaperonins CCT7 and CCT8 and early response genes EGR3 and IER5, were all upregulated in both dependent and nondependent base regions. The increased expression of all these genes was significantly greater in the nondependent regions (2.3- to 6.5-fold) compared with dependent regions (1.0- to 1.7-fold) (Fig. 5). In keeping with the reported protective and anti-inflammatory actions of these proteins (55, 58), expression of cytokines (TNF-α, IL-2, IL-6 receptor, and IL-7) and chemokines (IL-8, CCL, and CXCL families) was decreased in the nondependent regions (−1.1- to −2.0-fold), whereas it increased in dependent regions (1.0- to 2.0-fold). Thus, in the dependent regions of the lung base, which are flooded and collapsed with minimal penetration of air throughout the respiratory cycle (Fig. 2), there is an enhanced inflammatory response. In contrast, the well-aerated and potentially overdistended nondependent regions experience a robust stress response along with reduced cytokine and chemokine expression. These disparate responses are consistent with the results of a recent study in rats in which bacterial endotoxin plus injurious ventilation revealed a similar inverse relationship between HSP70 levels and IL-6 and IL-1β mRNA expression (56). Studies examining the time course of these changes and further modulating mechanical conditions will help determine how these factors contribute to protection vs. injury in VALI.

To further illustrate the significance of these regional differences in gene expression, we compared our list of genes differentially regulated between dependent and nondependent regions of the lung base with a list of candidate genes identified from the literature (3, 10, 13, 22, 24, 29, 35, 60). Of the ~226 ALI candidate genes identified, 87 (including closely related genes in the same family) met our criteria for significantly differentially changed between dependent and nondependent regions. Remarkably, expression of 19 of these genes were changed in opposite directions, mostly upregulated in the dependent regions while downregulated in the nondependent region of the same lung in the same animal. Thus, the particular pattern of gene expression in the injured lung is critically dependent on the sampling location. Whether these regional differences are due to different mechanical stresses, the population of cell types in these regions or some other aspect of the local physiology such as perfusion remains unknown. It should be apparent, however, that sampling approaches that average expression patterns across both apex and base, dependent and nondependent regions are likely to under-estimate the impact of genes that exhibit regional-specific expression. Averaging out of regional differences may explain the increasing numbers of differentially regulated genes detected as the regions examined become more localized. Furthermore, insight into the earliest cellular responses constituting VALI, and the contribution of different regional mechanical phenomena to these responses (that may be modified by patient and ventilator management), must be sought in the context of the mechanical heterogeneity that characterizes human ALI.

In summary, we have demonstrated significant differences in gene expression between different regions of the lung in a canine model of ALI, regions that undergo very different mechanical stresses during mechanical ventilation. These findings have important implications for the design and interpretation of studies investigating the mechanisms of VALI. A hallmark of human ALI is the heterogeneity of tissue involvement, and management strategies that reduce heterogeneity, in particular extremes of mechanical behavior, are the focus of current clinical investigations. Correlating regional cellular responses with the local mechanical and biological milieu may help us to not only understand how different mechanical stresses cause or propagate existing lung injury but also identify therapeutic targets, associate specific biomarkers with different aspects of evolving injury, and find surrogate endpoints to better predict outcomes and guide therapy.

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