Differential gene profiling in acute lung injury identifies injury-specific gene expression*

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Objectives: Acute lung injury can result from distinct insults, such as sepsis, ischemia-reperfusion, and ventilator-induced lung injury. Physiologic and morphologic manifestations of disparate forms of injury are often indistinguishable. We sought to demonstrate that acute lung injury resulting from distinct insults may lead to different gene expression profiles.

Design: Microarray analysis was used to examine early molecular events in lungs from three rat models of acute lung injury: lipopolysaccharide, hemorrhage shock/resuscitation, and high-volume ventilation.

Setting: University laboratory.

Subjects: Male Sprague-Dawley rats (body weight, 300–350 g).

Interventions: Rats were subjected to hemorrhagic shock or lipopolysaccharide followed by resuscitation or were subjected to sham operation. First hit was followed by ventilation with either low (6 mL/kg) or high (12 mL/kg) tidal volume for 4 hrs.

Measurements and Main Results: Physiologic and morphologic variables were assessed. Total RNA was hybridized to Affymetrix chips. Bioconductor was used to identify significantly altered genes. Functional enrichment predictions were performed in Gene Ontology Tree Machine. Confirmation stud-

ies included real-time polymerase chain reaction, Western blots, and immunohistochemistry. Physiologic and morphologic variables were noncontributory in determining the cause of acute lung injury. In contrast, molecular analysis revealed unique gene expression patterns that characterized exposure to lipopolysaccharide and high-volume ventilation. We used hypergeometric probability to demonstrate that specific functional enrichment groups were regulated by biochemical vs. biophysical factors. Genes stimulated by lipopolysaccharide were involved in metabolism, defense response, immune cell proliferation, differentiation and migration, and cell death. In contrast, high-volume ventilation led to the regulation of genes involved primarily in organogenesis, morphogenesis, cell cycle, proliferation, and differentiation.

Conclusions: These results demonstrate the application of functional genomics to the molecular “fingerprinting” of acute lung injury and the potential for decoupling biophysical from biochemical injury. (Crit Care Med 2008; 36:855–865)

KEY WORDS: transcriptional profiling; acute respiratory distress syndrome; ventilator-induced lung injury; bioinformatics; sepsis; shock

Acute respiratory distress syndrome (ARDS), the most severe manifestation of acute lung injury (ALI), is clinically defined as severe dysfunction of gas exchange and chest radiographic abnormalities after a predisposing injury in the absence of heart failure (1). Despite advances in life support, an unacceptably

high mortality persists (25–50%) (2, 3). It has become apparent that mechanical ventilation itself can be injurious to the lung (i.e., ventilator-induced lung injury) (4). Thus, elucidating the molecular characteristics of ALI/ventilator-induced lung injury is vital to the development of novel approaches for diagnosing and managing ARDS.

Regardless of the pathogenesis, the clinical manifestations of ARDS are indistinguishable from the original insult. Therefore, a common pathway of acute inflammatory responses has been proposed to explain how different insults may lead to lung tissue damage, deterioration of oxygenation function, and subsequent dysfunction in multiple organs.

Iatrogenic injury associated with the supportive care of the ALI/ARDS patient and the failure of anti-inflammatory therapies with specific molecules, such as tumor necrosis factor- α binding protein, have contributed to the pressing need for novel approaches in the management of this syndrome in the intensive care unit (5, 6). There is increasing awareness that injury-specific mechanisms and individualized therapies for ARDS patients may become attractive therapeutic options in

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the future (7). To this end, specific mechanisms of injury in the lung must be elucidated.

In the present study, we explored the hypothesis that ALI is not a stereotyped response of the lung to injury but rather a composite of specific responses to different coexisting injury mechanisms. We postulated that exploring the global response to injury using microarray technology would reveal the presence of injury-specific differential gene expression patterns in comparable lung injury models. We also proposed that such patterns would contain genes that are biologically plausible and functionally related and thus can be exploited for future mechanistic studies and, more importantly, may serve as a template for novel molecular diagnostics of ALI.

Whole-genome approaches have been proposed as feasible and efficient ways of dissecting the molecular response to injury. Stimulus-specific co-expression patterns describing the transcriptional behavior of multiple genes simultaneously in response to an insult may provide clues to the underlying molecular mechanisms of injury; these injury-specific patterns could also be used as “molecular

signatures” for clinical diagnosis and prognosis (7). For example, multiple CXC chemokines up-regulated by tumor necrosis factor- α in human lung epithelial cells are located closely on the same chromosome (8). A group of transforming growth factor- β -inducible genes is up-regulated together in bleomycin-treated animals (9). Using genetic linkage analysis, a pulmonary irritant-sensitive locus on murine chromosome 6 was identified (10). Inspired by these investigations, the objective of the present study was to explore whether injury-specific differential gene expression patterns could be recognized and separated from genes that are commonly involved in ALI from comparable and otherwise indistinguishable animal models of lung injury.

In the present study, we used adult rats treated with lipopolysaccharide (LPS; model of septic shock) or hemorrhagic shock/resuscitation (HS; model of ischemia/reperfusion) as the “first hit,” followed by mechanical ventilation as the “second hit” to test our hypothesis that ALI from different causes can lead to specific molecular profiles based on injury-specific differential gene expressions.

METHODS

Animal Experiments. Male Sprague-Dawley rats ($n = 24$, 300 to 350 g; Charles River, Montreal, Quebec, Canada) were anesthetized with ketamine hydrochloride (80 mg/kg; Ayerst Veterinary Laboratories, Guelph, Canada) and xylazine (8 mg/kg; Bayer, Toronto, Canada) administered intraperitoneally. After tracheostomy, a 14-gauge catheter was inserted into the trachea. The right carotid artery was cannulated with a 24-gauge Angiocath (Becton Dickinson, Franklin Lakes, NJ) for measuring mean arterial blood pressure (MAP), blood withdrawal, and resuscitation. The tail vein was catheterized with a 22-gauge Angiocath (Becton Dickinson) for continuing sedatives of ketamine hydrochloride ($20 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{hr}^{-1}$), xylazine ($4 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{hr}^{-1}$), and pancuronium ($0.3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{hr}^{-1}$). Hemodynamics, lung elastance, and arterial blood gas measurements were performed (11). Animals in all groups underwent the same initial instrumentation and anesthesia. Rats were randomized to receive HS (volume reduction to MAP of 40 mm Hg for 30 mins) or systemic administration of LPS (5 mg/kg intravenously) followed by resuscitation or to undergo sham operation (without HS or LPS). Animals were subsequently randomized again to receive mechanical ventilation with either a low tidal volume (LV) of 6 mL/kg and 5 cm H_2O of positive end-expiratory pressure or a high tidal volume

Figure 1. Physiologic variables of acute lung injury. Animals received lipopolysaccharide (LPS; 5 mg/kg intravenously) or hemorrhagic shock (HS) followed by resuscitation, or received sham operation (Sham), and were then subjected to mechanical ventilation with low (LV) or high (HV) tidal volume as described in the Methods section. Mean arterial pressure (MAP) (a), PaO_2 (b), and pulmonary elastance (c) were measured before and during the experiment; lung wet/dry weight ratios (d) were determined at the end of the experiment. All values are expressed as mean $\pm$ SD ($n = 4$). BL, baseline. $*p < .05$ and $**p < .01$ vs. ShamLV; $+p < .05$ vs. HSLV; $\hat{p} < .05$ vs. LPSLV. e, Hematoxylin and eosin staining of the lungs shows no dramatic pathologic changes that can be differentiated by pathologic examination. Magnification, $\times 400$.

(HV) of 12 mL/kg without positive end-expiratory pressure for 4 hrs. At the completion of the experiment, animals were killed and the lung tissues were processed for microarray (left lung) or for assessments of histology, immunohistochemistry, and ELISA (right lung), as described previously (11). All animals received care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research, the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication 85-23, revised 1985), and the *Guide to the Care and Use of Experimental Animals* formulated by the Canadian Council on Animal Care. The experimental protocol was approved by our Institutional Animal Care and Use Committee.

Microarray and Data Analysis. Total RNA was extracted from the left lung using Trizol reagent (Invitrogen, Burlington, Ontario, Canada) and purified with RNeasy (Qiagen, Chatsworth, CA). Equal amounts of RNA from four animals in each group was pooled for microarray (12). REA 230 plus 2.0 chips (31,099 expressed sequences) from Affymetrix (Santa Clara, CA) were used. Intensity values were determined using MAS 5.0 (Affymetrix). Complete microarray data set and experimental protocol have been submitted to the National Center for Biotechnology Information Gene Expression Omnibus according to the Minimum Information About a Microarray Experiment (MIAME) standard for microarray data (GSE 4770). Scaled values were analyzed in Genespring (<http://www.silicongenetics.com>). Quantile normalization was performed. Gene expression data were further filtered based on raw intensity value. Genes with raw intensity values that did not meet a minimum threshold (more than 50 in at least three out of six of the experimental conditions) were excluded from the analysis. To identify injury-specific differential gene expression, scaled, summarized, and normalized data were imported into R (Statistical Package for Microarray Analysis: <http://www.statistik.lmu.de/~strimmer/notes/rexpress.html>), analyzed using the Linear Models for Microarray Data library (LIMMA) (13) of the Bioconductor package (14) (Bioconductor, <http://www.bioconductor.org>), and confirmed using Significance Analysis of Microarray (SAM, <http://www-stat.stanford.edu/~tibs/SAM>).

Hierarchical clustering was used to build condition and gene trees for differential gene expression, and K-means was used to plot subgroups of genes with similar expression patterns (Genespring). Predictions regarding functional enrichment were performed with Gene Ontology (GO) Tree Machine (<http://bioinfo.vanderbilt.edu/gotm/>) and Onto-Express (<http://vortex.cs.wayne.edu/projects.htm>). Information regarding specific putative functions was obtained from SOURCE (<http://source.stanford.edu/cgi-bin/source/sourceSearch>) and the National Center for

Biotechnology Information database (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=gene>).

Confirmation Studies. To confirm the results from microarray studies, >30 genes were tested with RNA from each individual animal for quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR), as previously described (8, 15). The protein levels of several inflammatory mediators (tumor necrosis factor- α , interleukin-1 β , pentraxin 3) were measured by enzyme-linked immunosorbent assay (8, 11, 16). Protein expression of tissue factor in the lung was determined with immunohistochemistry staining (protocol is available on request. Correlation between changes in tissue factor messenger (m)RNA levels and lung injury variables (elastance, Pao_2 , and wet-to-dry ratio) were calculated. Graphs and tables displaying individual gene expression values and statistical analysis for qRT-PCR were performed with JMP (statistical analysis software, <http://www.jmp.com>) and Excel (<http://www.excel.com>).

All gene symbol abbreviations used in this article are spelled out in a list available in the online supplemental material.

RESULTS

ALI Induced by HS, LPS, and HV. To identify injury-specific molecular patterns of expression, animals were randomized into three distinct rat models of ALI. To introduce HS, animals were bled to reduce MAP to 40 mm Hg within the first 15 mins. The low MAP was maintained for another 15 mins, followed by resuscitation (Fig. 1a). In animals that received LPS, MAP fell gradually within the first 1.5 hrs to approximately 60 mm Hg; this was followed by resuscitation with crystalloids (Fig. 1a). Sham-operation animals received identical anesthesia and surgery protocols. After adequate resuscitation (MAP of ≥ 80 mm Hg), all animals were randomized into two subgroups and ventilated with two clinically applicable ventilation regimens, either HV or LV for 4 hrs. The decrease in MAP only reached statistical significance in the LPSHV and HSHV groups at the end of the experiment in comparison with

Figure 2. Differential gene expression profiles induced by acute lung injury. *a*, Hierarchical clustering. The condition tree ($n = 16,599$) shows that gene expression patterns cluster into two primary groups. Gene expression in the low tidal volume ventilation (LV) hemorrhagic shock (HS) group (HSLV) is closer to the baseline (sham operation [Sh]) group (ShLV). In contrast, a different gene expression profile is primarily related to either lipopolysaccharide (LPS) exposure or high tidal volume ventilation (HV). *b*, Venn diagram (generated in Genespring) showing intersection between gene lists identified as being LPS, HV, or HS specific. *c*, LPS-specific genes ($n = 885$); *d*, HV-specific genes ($n = 232$); *e*, HS-specific genes ($n = 301$). The logarithmic scales of normalized row intensities were plotted against the experimental conditions. The changes of the genes are expressed in red for up-regulation and in green for down-regulation. LPS- or HV-dependent expression patterns are revealed by K-means plots. In contrast, HS-specific genes do not show consistent patterns.

that in the ShamLV group ($p < .05$) (Fig. 1a). PaO_2 was significantly decreased in all HV groups compared with that in the ShamLV group (Fig. 1b). Pulmonary compliance deteriorated in animals ventilated with the HV strategy compared with that of the ShamLV group at the end of 4 hrs of mechanical ventilation (Fig. 1c). Measurement of wet-to-dry lung weight ratios showed that LPS alone significantly increased water content in the lung, and animals ventilated with the HV strategy had a significantly greater lung water compared with their counterparts ventilated with the LV strategy ($p < .05$) (Fig. 1d). Histologic assessment of ALI scores was performed in ten randomly selected fields per animal, in four animals per group, at a magnification of $\times 400$ in a blinded fashion. Animals randomized to the HV strategy had increased neutrophil infiltration, diffuse alveolar damage, and hemorrhage (Fig. 1e), but these pathologic changes cannot be differentiated among the three HV ventilation groups at 4 hrs ($p > .05$).

Gene Profiling of ALI. All six groups (ShamLV, ShamHV, LPSLV, LPSHV, HSLV, and HSHV) were used in microarray analysis. Gene expression profiles were determined using gene chips containing 31,099 oligonucleotide probe sets, each representing an expressed sequence, corresponding to more than half of the protein coding capacity of the rat genome. Of these, 19,756 were deemed as expressed in the lung (63.5%). After filtering for quality of expression, 16,599 "present" expressed sequences were selected for further analysis. Hierarchical clustering was used to visualize the global transcriptional profiles of genes selected as present. Biological interpretation of hierarchical clustering suggests that gene expression profiles can be divided into two distinct groups: (a) low injury group (ShamLV and HSLV) and (b) high or higher injury group (samples that received HV or LPS). The latter is further composed of two subgroups based on whether animals were exposed to LPS or to HV (Fig. 2a).

To identify injury-specific gene expression patterns, scaled, summarized, and normalized data were imported into R (Statistical Package for Microarray Analysis) and analyzed using the Linear Models for Microarray Data library (13) of the Bioconductor package (16). We performed multivariate analysis of variance. In this analysis, we assumed that all factors (LPS, HS, and HV) affect gene ex-

pression equally and used the same estimates of error for all three conditions on the same gene. In each case, the three effects estimated were considered "orthogonal" to each other (i.e., completely independent). In each case, we allowed for 2 degrees of freedom for error. Benjamini and Hochberg (17) multiple-testing correction was applied. Genes were selected based on Bayes Linear Models for Microarray Data model and false discovery rate, ranked as being differentially expressed in decreasing order of unadjusted p values. We chose to select only top genes based on the cut-off value of $p \leq .01$. Using this strategy, we selected 1,013 LPS-responsive genes; 380 HS-responsive genes, and 332 HV-responsive genes. A Venn diagram of gene lists was generated in Genespring (Fig. 2b).

Genes that were up- or down-regulated by LPS (Fig. 2c) or HV ventilation (Fig. 2d) can be clearly identified by their patterns of expression. In contrast, plotting of the 301 genes whose expression may be related to exposure to HS revealed no recognizable pattern (Fig. 2e).

Identification of LPS-Specific Gene Expression Patterns. To facilitate visualization of expression patterns of genes selected as LPS responsive, we plotted the gene expression values using K-means, a way to plot gene expression with normalized raw intensity values (Fig. 3a). Three sets of subgroup genes were identified. Set 1 contains 197 genes that are down-regulated by LPS exposure. Set 2 is close to the mirror image of set 1 and contains 458 genes that are up-regulated by LPS. Set 3 contains 358 genes whose expression is usually low in the control condition (ShamLV). Exposure to LPS leads to up-regulation of these genes, and exposure to HV ventilation seems to be additive to LPS at the gene expression level.

Multiple genes from each K-means set were measured with qRT-PCR to confirm these gene expression patterns. The qRT-PCR results of sialoporphin (Spn1), small inducible cytokine B11 precursor (Cxcl11), small inducible cytokine A20 precursor (Scya20/CCL20), and cytokine-inducible SH2-containing protein (Cish1), also known as suppressor of cytokine signaling (Socs-1), are shown (Fig. 3c) as

Figure 3. Lipopolysaccharide (LPS)-specific gene expression pattern. *a*, K-means plot of 1,013 genes identified as responsive to LPS. *Set 1* contains genes that are down-regulated by LPS. *Set 2* and *set 3* contain genes whose expression is up-regulated by LPS. *b*, Representative genes are shown as examples as normalized raw intensity value by microarray. *c*, Changes of these genes were confirmed by quantitative real-time reverse transcriptase-polymerase chain reaction, expressed as the ratio to the housekeeping gene HMBS. Values are mean $\pm$ SD from four samples: *Spn*, sialoporphin, gpL115 leukosialin CD43; *Cxcl11*, chemokine C-X-C motif ligand 11; *Scya20*, small inducible cytokine subfamily A20; *Cish1*, cytokine-inducible SH2-containing protein 1. LV, low tidal volume ventilation; HV, high tidal volume ventilation; Sham, sham operation; HS, hemorrhagic shock.

examples to demonstrate similar expression patterns in comparison with microarray data (Fig. 3b). By matching UniGene or GenBank, or both, numbers, a group of genes reported in other models of LPS-induced ALI (18, 19) was also identified in the present study (Table 1).

To further determine the feature of LPS-related genes, we searched for enrichment in predicted functions among genes that shared this expression pattern. This was carried out using Onto-Express and GOTree Machine, which are united by their use of the GO database provided by the GO Consortium. A broad functional classification by biological process and molecular function of the genes, in which expression was altered by exposure

to LPS, was performed. Using hypergeometric probability (20, 21), the likelihood (or *p* value) that a functional enrichment could have occurred by chance alone after exposure to LPS was calculated. A total of 226 GO categories were identified as relatively enriched (*p* < .01) by exposure to LPS. Specific terms for functionally enriched biological processes were extracted from multiple levels within the GO hierarchy and ranked by *p* value so that only the top 50 significant categories are listed in supplemental eTable 1. This broad classification includes genes encoding for metabolism, defense response, immune response, immune cell proliferation, differentiation, and migration, cell adhesion, motility, and cell death.

Directed acyclic graph (<http://bioinfo.vanderbilt.edu/gotm/>) view of the significantly enriched GO categories (*n* = 1013; *p* < .01) related to physiologic process among LPS-regulated genes are shown in supplementary eFigure 1. The most significantly altered genes involved in this functional category are displayed using hierarchical clustering so that genes contained in set-specific examples are highlighted (supplementary eFigure 1).

Of the 1,013 genes selected as LPS responsive, 418 are EST clones. Although the function of these transcripts is unknown and some of them could be pseudogenes, the LPS-specific regulation of these transcripts suggests that they may

Table 1. Expression pattern of genes that were also identified in other models of lipopolysaccharide (LPS)-induced acute lung injury (18, 19)

Gene Symbol	Genbank	UniGene	Gene Name	LPSLV/ShamLV	LPSHV/ShamLV
CCL2	NM.031530	Rn.4772	Chemokine (C-C motif) ligand 2	2.02	2.25
Ccl22	AF432871	Rn.48727	Chemokine (C-C motif) ligand 22	2.59	3.34
Ccl22	AF163477	Rn.48727	Chemokine (C-C motif) ligand 22	5.25	7.23
CCL3	BE095824	Rn.7857	Chemokine (C-C motif) ligand 3	0.64	0.73
CCL4	U06434	Rn.37880	Chemokine (C-C motif) ligand 4	2.70	2.95
CCL7/MCP3	BF419899	Rn.26815	Chemokine (C-C motif) ligand 7	2.34	4.91
Ccr2	NM.021866	Rn.44347	Chemokine (C-C motif) receptor 2	0.52	0.66
Cd14	NM.021744	Rn.42942	Monocyte differentiation antigen CD14	1.54	1.94
Cd44	NM.012924	Rn.1120	CD44 molecule (Indian blood group)	1.51	1.73
Gm1960	D87927	Rn.10525	Gene model 1960, (NCBI)	5.08	8.99
Gm1960	D87927	Rn.10525	Gene model 1960, (NCBI)	5.78	11.62
Gm1960	D87927	Rn.10525	Gene model 1960, (NCBI)	4.61	7.79
Cish1	BE100794	Rn.82754	Cytokine inducible SH2-containing	2.30	2.58
Cish3	NM.053565	Rn.29984	Cytokine inducible SH3-containing	1.32	1.63
Cx3cl1	NM.134455	Rn.4106	Chemokine (C-X3-C motif) ligand 1	2.42	2.68
Cxcl10	U22520	Rn.10584	Chemokine (C-X-C motif) ligand 10	1.63	1.70
CXCL13	AA892854	Rn.6917	Chemokine (C-X-C motif) ligand 13	2.16	1.76
Fcer2a	AI549450	Rn.43990	Fc receptor, IgE, low affinity II, alp	2.85	2.57
Fosl1	NM.012953	Rn.11306	FOS-like antigen 1	2.21	3.73
CXCL1	NM.030845	Rn.10907	Chemokine (C-X-C motif) ligand 1	1.78	1.91
			nuclear factor of kappa light polypeptide gene		
NFKBIA	AA996726	Rn.23000	Enhancer in B-cells inhibitor, alpha	1.80	1.54
Il1a	NM.017019	Rn.12300	Interleukin 1, alpha	2.11	1.99
Il1r1	NM.013123	Rn.9758	Interleukin 1 receptor, type I	1.16	1.52
Il1m	NM.022194	Rn.44376	Interleukin 1 receptor antagonist	2.46	1.90
Il6	NM.012589	Rn.9873	Interleukin 6 (interferon, beta 2)	2.91	4.06
Irf1	NM.012591	Rn.6396	Interferon regulatory factor 1	1.54	1.85
Irf7	BF411036	Rn.6246	Interferon regulatory factor 7	2.66	2.93
			Interferon dependent positive acting		
Isgf3g	AI029121	Rn.18149	Transcription factor 3 gamma	1.78	1.54
			Chemokine (C-X-C motif) ligand 5/CXC		
Cxcl5/LIX	NM.022214	Rn.44449	Chemokine LIX chemokine (C-X-C motif) ligand 9/monokine	22.11	39.34
CXCL9/Mig	AI044222	Rn.7391	Induced by gamma interferon	7.67	7.78
Mx1	X52711	Rn.10373	Myxovirus (influenza virus) resistance 1	3.40	4.02
Orm	NM.053288	Rn.10295	Orosomucoid 1	7.04	9.08
Pail	AI500951	Rn.107899	Plasminogen activator inhibitor, type I	5.75	8.60
			Avian reticuloendotheliosis viral (v-rel)		
RelB	BF408850	Rn.40873	Oncogene related B	1.53	1.68
Saa3	BF282318	Rn.98299	Serum amyloid A 3	3.05	3.46
Slpi	NM.053372	Rn.18560	Secretory leukocyte peptidase inhibitor	1.39	1.68
Stat1	AW434718	Rn.33229	Signal transducer and activator of transcription 1,91kDa	1.97	1.92
Stat1	NM.032612	Rn.33229	Signal transducer and activator of transcription 1, 91kDa	2.11	2.28
Tfec	L08812	Rn.9659	Transcription factor EC	1.69	1.49
TNFaip2	BI278479	Rn.7779	Tumor necrosis factor, alpha-induced protein 2	2.66	2.66

LV, low tidal volume ventilation; Sham, sham operation; HV, high tidal volume ventilation.

Figure 4. High tidal volume ventilation (HV)–responsive genes. *a*, K-means plot of 332 genes that fulfilled criteria for selection as HV-responsive genes. *Sets 1* and *2* contain genes up-regulated by HV. Genes in *set 3* were down-regulated by HV. *b*, Normalized raw intensity values by microarray for selected genes are plotted as examples; *c*, changes in gene expression of the same group of genes were confirmed by quantitative real-time reverse transcriptase–polymerase chain reaction expressed as the ratio to the housekeeping gene HMBS: *Hmox*, heme oxygenase 1; *Scya17*, small inducible cytokine subfamily A (Cys-Cys) member 17 (CCL17); *Lrp4*, low-density lipoprotein receptor–related protein 4. LV, low tidal volume ventilation; Sham, sham operation; LPS, lipopolysaccharide; HS, hemorrhagic shock.

be potential markers to monitor the LPS-related gene expression in the lung. Detailed information regarding putative biological functions of the top 30 selected genes in each set is presented in supplemental eTable 2.

Identification of HV-Related Gene Expression Patterns. To enable visualization of the transcriptional expression pattern that we have identified as ventilation specific, K-means plotting was used to show the normalized raw intensity values. Three main patterns of expression are associated with exposure to high tidal volume. Set 1 and set 2 (Fig. 4a) included 118 and 66 genes, respectively, that were up-regulated by HV ventilation. The difference in these two sets lies in the putative additive response seen with LPS. Set 3 identifies genes that were down-regulated by HV ventilation (Fig. 4a). Real-time qRT-PCR was used to confirm set-specific gene expression patterns. Heme-oxygenase (Hmox), small inducible cytokine subfamily A (Cys-Cys) member 17 (Scya17), and

low-density lipoprotein receptor–related protein 4 (Lrp4) are shown as examples of similarity between microarray and qRT-PCR results (Fig. 4, *b* and *c*). Table 2 shows fold change in expression of genes from this study, identified as significantly altered in a separate cross-species study of ventilator-induced (large tidal volume) lung injury (22) and a study of early stress response in ALI (23).

Predicted functional enrichment among HV-sensitive genes shows that a total of 88 GO categories were relatively enriched ($p < .01$). Major significant functional categories include genes involved in morphogenesis, organogenesis, healing and repair, cell cycle progression, proliferation (fibroblasts), and differentiation (keratinocytes). Of specific note is enrichment for catecholamine biosynthesis and dopamine metabolism (supplemental eTable 3).

Directed acyclic graph view of the significantly enriched GO categories ($p < .01$) related to physiologic process among HV-regulated genes is shown in

supplementary eFigure 2. The top significantly altered genes involved in this functional category are displayed using hierarchical clustering so that set-specific examples are highlighted (supplemental eFigure 2).

Detailed information regarding putative biological functions of the top 30 selected genes in each set is presented in supplemental eTable 4. Of specific interest is the identification of five members of the keratin family (cytokeratin 21, 8, and 14 and keratin complex, acid genes 18 and 19) and other structural components of the cell that are known to co-localize to focal adhesion kinase and adherens junction (son of sevenless 2 [Sos2], Src, and parvin), tight junctions (claudins and villin 2), tubulin β 1 (TBB1), and laminin β 2 (LAMC2) (supplemental eTable 4).

Confirmation Studies of Microarray Data with Other Measures. Expression and distribution of tissue factor in the lung are used as an example to show our confirmation studies. The expression patterns of

Table 2. Expression pattern of genes that were also identified in a separate cross-species study of ventilator-induced lung injury (22) and a study of early stress response in acute lung injury (23)

Gene Symbol	Genbank	UniGene	Gene Name	HSHV/ ShamLV	LPSHV/ ShamLV	ShamHV/ ShamLV
Atf3	NM.012912	Rn.9664	Activating transcription factor 3	1.81	2.48	1.74
Areg	NM.017123	Rn.10568	Amphiregulin	3.14	1.89	2.70
Arg2	NM.019168	Rn.11055	Arginase 2	1.53	1.58	1.13
F3	NM.013057	Rn.9980	Thromboplastin, tissue factor	2.41	2.81	2.54
Cyr61	NM.031327	Rn.22129	Cysteine-rich, angiogenic inducer, 61	5.68	0.79	0.51
Egfr	M37394	Rn.37227	Epidermal growth factor receptor	1.85	3.59	1.25
Gja1	BG378227	Rn.10346	Gap junction protein, alpha 1, 43kDa	1.87	3.20	2.09
Gclm	NM.017305	Rn.2460	Glutamate-cysteine ligase modifier subunit	1.20	1.50	1.22
Gadd45a	NM.024127	Rn.10250	Growth arrest and DNA-damage-inducible, alpha	1.49	2.80	1.66
Gch	NM.024356	Rn.28195	GTP cyclohydrolase 1	1.19	2.17	1.24
Gbp2	NM.133624	Rn.25736	GTP-binding protein homolog	1.21	1.79	1.38
Hspa1a	NM.031971	Rn.1950	Shock 70kD protein 1A	2.45	1.75	3.48
Ifrd1	NM.019242	Rn.3723	Interferon-related developmental regulator 1	1.68	1.62	1.59
Il1a	NM.017019	Rn.12300	Interleukin 1, alpha	0.94	1.99	0.66
Il1r2	NM.053953	Rn.10758	Interleukin 1 receptor, beta; type II receptor	1.83	1.98	2.16
Il6	NM.012589	Rn.9873	Interleukin 6 (interferon, beta 2)	2.67	4.06	2.51
Mmp9	NM.031055	Rn.10209	Matrix metalloproteinase 9	0.32	0.27	0.47
Mt1a	AF411318	Rn.54397	Metallothionein 1A	2.57	3.32	2.82
Plaur	AF007789	Rn.82711	Plasminogen activator, urokinase receptor	1.44	1.59	1.53
Ptgs2	U03389	Rn.44369	Prostaglandin-endoperoxide synthase 2	1.75	4.61	1.30
Pai1/Serpine1	NM.012620	Rn.29367	Plasminogen activator inhibitor, type I/serine (or cysteine) proteinase inhibitor, clade E, member 1	5.41	6.90	3.79
Pai1/Serpine1	AI500951	Rn.107899	Plasminogen activator inhibitor, type I/serine (or cysteine) proteinase inhibitor, clade E, member 2 chemokine (C-C motif) ligand 2/ Monocyte	5.72	8.60	3.85
Ccl2/MCP1	NM.031530	Rn.4772	Chemotactic factor 1/small inducible cytokine A2	1.66	2.25	1.52
Scd1	J02585	Rn.1023	Stearoyl-coenzyme A desaturase 1	2.72	1.10	3.44

HS, hemorrhagic shock; HV, high tidal volume ventilation; Sham, sham operation; LV, Low tidal volume ventilation; LPS, lipopolysaccharide.

tissue factor mRNA are very similar between the microarray and qRT-PCR results (Fig. 5, *a* and *b*). Protein expression of tissue factor was examined with immunohistochemistry. Increased staining of tissue factor in HV groups can be seen in comparison with groups subjected to LV (Fig. 5c). More importantly, changes in tissue factor mRNA expression levels correlated significantly with the degree of lung injury as measured by physiologic markers of pulmonary dysfunction: elastance, Pao₂ levels, and wet-to-dry ratio (Fig. 5, *d* and *e*). The mRNA and protein levels of tumor necrosis factor- α , interleukin-1 β , and pentraxin 3 (a new inflammatory mediator identified (16, 24)) were also measured with qRT-PCR and enzyme-linked immunosorbent assay, respectively. These inflammatory mediators are rapidly induced in all injury groups. As inducible genes, their protein levels were up-regulated with similar patterns as their gene expressions. We previously determined that pentraxin 3 expression and distribution in the lung were increased as determined by immunohistochemistry, further confirming our current findings (11).

DISCUSSION

The current report significantly extends previous studies aimed at understanding the molecular features of early ALI by demonstrating that early lung injury can be distinguished on the basis of specific transcription profiles (18, 19, 22, 23). In keeping with clinical observations, we found that physiologic and pathologic studies cannot distinguish the type of lung injury across different models. In contrast, the transcriptional profiles identified with microarray and bioinformatics enabled the decoupling of biochemical from biophysical injury. Detailed analysis of co-transcriptional profiles revealed a group of LPS-specific genes and a group of high-volume ventilation-specific genes. Importantly, these unique gene expression patterns are associated with specific functional enrichment (Fig. 6).

With the rapid development of microarray technology and bioinformatics, several groups have taken the advantages of whole-genome approaches to identify individual molecules that may play a role in ALI. Although specific gene expression patterns

have been noted during data analyses, confirmation of the importance of these genes has been a challenge. Many researchers have focused on one or two genes after screening thousands of transcripts. This approach provides a definite answer regarding the importance of a particular gene, but numerous genes associated with the pathologic process of ARDS are lost—buried during data mining. The approach used in the present study underscores the importance of co-differential expression as a determinant of biological significance.

We analyzed early events in three distinct rat models of lung injury: ischemia-reperfusion, systemic LPS administration, and injurious mechanical ventilation. The routine and accepted physiologic and histologic strategies were sufficient to discern between groups of animals that had received HV ventilation from those that received LV ventilation. However, it was insufficient to discriminate based on these pathophysiologic variables the underlying pathogenesis of the ALI. In contrast, analysis of gene co-expression patterns enabled the identification of injury-specific expression patterns. Compared with the

Figure 5. Confirmation of microarray data with tissue factor (TF) as an example. The messenger (m)RNA levels of TF were measured with microarray (a) and quantitative real-time reverse transcriptase-polymerase chain reaction (b), respectively. Increased TF protein in the lung tissue was revealed by immunohistochemistry staining (c). Correlation between TF mRNA expression levels and physiologic data: % Elastance (d), wet-to-dry ratios (e), and PaO₂ (f). LV, low tidal volume ventilation; HV, high tidal volume ventilation; Sham, sham operation; HS, hemorrhagic shock; LPS, lipopolysaccharide.

complex clinical manifestations of ARDS, animal models only represent the schematic feature of this syndrome. However, these simplified models allow us to compare and define the profiles of genes associated with known insults. To focus on early transcriptional events of gene ex-

pression, we only used 4 hrs of ventilation. Although this does not detract from the identification of injury-specific patterns of injury, further studies with multiple time points during ALI will be necessary. To identify the contribution of major cell types in the lung for these

gene expression patterns, cell culture studies should also be considered. Post-transcriptional modification of mRNA cannot be ruled out as a potential mechanism for the alteration in message abundance in our *in vivo* experiment. One may argue that the dramatic changes in gene

Figure 6. Enrichment in predicted Gene Ontology biological functions. Ratio of observed vs. expected functional enrichment was calculated for all categories identified as significantly enriched ($p = .01$) in lipopolysaccharide (LPS)- or high tidal volume ventilation (HV)-specific genes. Top 20 categories as plotted: top LPS-predicted functional enrichment (a) and top HV-predicted functional enrichment (b). Detailed information generated in Gene Ontology Tree Machine is presented in online-only supplemental eTables 1 and 3.

expression *in vivo* could be mainly due to pulmonary cellular infiltration. Although that may be one of the underlying mechanisms, it does not undermine the existence of distinct molecular profiles that may be exploited, albeit for a brief period, for identifying different forms of injury. The presence of specific molecular patterns of injury will have to be validated in humans. Further studies may identify primary components of gene expression patterns that may be used to stratify patients and assess prognosis.

In this study, we relied on the differential expression of genes in two distinct

groups exposed to LPS to determine LPS-specific changes induced during the early phase of ALI. As a biological stimulator with well-defined signaling mechanisms, LPS influences the overall genome more dramatically than other insults. LPS can activate toll-like receptors and subsequently execute its signal through the nuclear factor- κ B pathway for transcriptional regulation (25). LPS also activates Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway *in vivo* (26). Indeed, among the LPS-specific genes identified, many were

predicted to exert a role in these pathways. Hypergeometric probability analysis identified that genes responsive to LPS were primarily involved in metabolism, defense response, immune response, immune cell proliferation, differentiation, and migration, cell adhesion, motility, and cell death (27). These findings fit the current knowledge that LPS-induced cell death (28) and inflammation is important for the evolution of ALI, and various genes identified in our model have also been highlighted as significantly altered in other models of LPS-induced lung injury (Table 1).

In comparison with the more thoroughly described LPS-related differential expression pattern, the set of genes regulated by HV ventilation has not been well defined. It is known that mechanical forces could activate signal transduction in lung cells (29, 30), which has been postulated as an underlying mechanism in ventilator-induced lung injury (31, 32). The unique feature of our study is that we exploit the heterogeneity of ALI to dissect out genes that are pathologically relevant to HV ventilation. The postulate is that, if a gene is truly involved in the pathologic process (i.e., HV ventilation), it will be differentially expressed in the presence of the stimulus of interest and in the company of other genes within its group, irrespective of changes in the first hit (e.g., LPS). We also used a model in which the minimum amount of injury (the LV ventilation groups) serves as baseline, enriching the data set for pathologic HV-related gene expression, rather than simply changing from normal, thus making the findings more clinically relevant. Top selected genes included SphK1. Data from animal studies have implicated sphingosine phosphate 1, the product of sphingosine phosphorylation by SphK1, in ALI (33, 34). Sphingosine phosphate 1 has been implicated in the barrier function in the lung (35, 36). It acts on five subtypes of G-protein-coupled receptors; these receptors are in turn coupled to different intracellular second messenger systems, including phospholipase C, phosphatidylinositol 3-kinase/protein kinase Akt, mitogen-activated protein kinases, and Rho- and Ras-dependent pathways (37). In our rat model, genes co-differentially expressed with SphK1 included phospholipase C beta 1, Rho guanosine triphosphatase-activating protein 8, and Akt2. Sphingolipids have also been implicated in keratin reorganization (38), regulation of plasminogen activator

inhibitor-1 expression (39), and disruption of claudins, integral organizers of tight junctions (34). As part of the HV differential expression pattern, we identified five keratin-related genes, two transcripts for plasminogen activator inhibitor-1, and three distinct types of claudins: 3, 4, and 7. These data suggest a functional correlation between co-expressed genes, with a central role for sphingolipids in HV-induced ALI. Moreover, cytoskeleton- and cell junction-related genes may be mechanosensitive and, consequently, play an important role in mechanotransduction (32). Alteration of these genes implicates a feedback between mechanotransduction and expression of related molecules. In addition to increasing gene expression levels, HV ventilation also suppressed a group of genes functionally enriched for signal transduction (Fig. 4a, set 3).

Both clustering analysis (Fig. 2a) and statistical analyses demonstrated that the effects of LPS or high-volume ventilation on gene expression are more effective than HS/resuscitation; this was intended to mimic ischemia/reperfusion as an important mechanism of injury, presumably priming the lung for subsequent injury via oxidative stress (40, 41). This may be explained by the fact that unlike LPS or HV, HS was not sustained throughout the experiment, correction of the hypotension may have returned the molecular profile to baseline, or the experimental condition was not powered to detect significant changes between HS injury and the baseline.

What do these injury-specific gene expression patterns mean to clinicians? First, this study clearly supports the concept that although a common molecular mechanism (or pathway) may indeed be involved in the pathogenesis of ARDS, injury-specific mechanisms cannot be ignored. Individual expression patterns indicate that each insult may have a specific effect on gene regulation at the genomic level. Second, expression profiles of a single gene or a few genes have been explored as biomarkers for clinical diagnosis and prognosis to guide clinical therapy. Results from this study suggest that injury-specific patterns should be considered as a potentially novel strategy for clinical diagnosis and prognosis (7). Moreover, injury-specific patterns of gene expression profiles provide novel targets for ARDS-related investigation and potential future clinical treatment options.

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