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Peripheral and Alveolar Cell Transcriptional Programs Are Distinct in Acute Respiratory Distress Syndrome

To the Editor:

Our understanding of transcriptomic responses in acute respiratory distress syndrome (ARDS) is derived, almost exclusively, from studies that have used samples procured from the peripheral circulation as opposed to the alveolar space (1–4). However, it is not known to what degree genomic signatures captured from circulating leukocytes accurately reflect the gene expression patterns of leukocytes in the lung. We hypothesized that the transcriptional signals from peripheral blood monocytes (PBMs) and alveolar macrophages (AMs) would be distinct in ARDS, and that alterations in the transcriptional state of immune cells in the lung would provide new insights into the pathogenesis of ARDS. Some of the results of these studies have been previously reported in the form of an abstract (5).

Methods

Subjects enrolled in the Phase II Randomized Placebo-controlled Trial of Omega-3 Fatty Acids for the Treatment of Acute Lung Injury trial (6) conducted between 2006 and 2008 were included in this study. We performed genome-wide expression analysis of total RNA isolated from paired AM and PBM samples purified from BAL fluid and peripheral blood specimens, respectively, collected from patients within 48 hours of the diagnosis of ARDS. Negative selection for AMs and PBMs was achieved by incubating cells with antibody-labeled microbeads containing the following markers: CD3, CD15, CD19, CD235a, CD294, and CD326. We did not use antibodies for CD294 (eosinophils) and CD326 (epithelial cells) for the blood samples because mononuclear cells were isolated from whole blood before antibody incubation via polyester gel centrifugation. RNA extracted from isolated cells was assessed for purity, and then hybridized to an Illumina HumanRef-8 BeadChip that was inclusive of 18,415 unique genes.

We performed variance stabilization and quantile normalization of the raw microarray data, using the Bioconductor package *lumi* (7). Detailed microarray information has been deposited at Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE89953>). Principal component analysis was performed on the basis of whole-genome gene expression variability between paired AM and PBM samples (8). Differential gene expression between the two cell populations was determined by a Bayesian implementation of the *t* test (<http://cybert.microarray.ics.uci.edu>) on log₂-transformed probe intensities, using a sliding window size of 101 and a Bayesian confidence estimate value of 10.

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Table 1. Patient Demographics and Clinical Characteristics

Characteristic	VFD High (n = 14)	VFD Low (n = 12)
Age, yr, mean ± SD	41 ± 18	43 ± 16
Female, n (%)	4 (29)	7 (58)
White, n (%)	10 (71)	12 (100)
ARDS risk factor, n (%)		
Sepsis	8 (57)	6 (50)
Trauma	6 (43)	6 (50)
Pneumonia	4 (29)	3 (25)
Other	2 (14)	2 (17)
Comorbidities, n (%)		
Diabetes	2 (14)	2 (17)
Cirrhosis	0 (0)	1 (8)
P/F ratio on admission, mean ± SD	217.3 ± 54.2	170.6 ± 61.9
VFD, median (mean ± SD)	23.5 (22.5 ± 2.4)	0 (1.2 ± 2.6)
APACHE II, mean ± SD	19.6 ± 6.2	20.9 ± 4.1
28-d mortality, n (%)	0 (0)	3 (25)

Definition of abbreviations: APACHE = Acute Physiology and Chronic Health Evaluation; ARDS = acute respiratory distress syndrome; P/F ratio = ratio of PaO₂ to FIO₂; VFD = ventilator-free days; VFD high = subjects with ≥18 VFD; VFD low = subjects with ≤7 VFD.

To identify transcriptional programs activated in AMs and PBMs in early ARDS and to determine their relationship with patient outcomes, we applied Gene Set Enrichment Analysis (GSEA), using 50 hallmark and 1,329 canonical

pathway gene sets curated from the Molecular Signature Database (MSigDB) (9). We used a false discovery rate threshold less than 0.05 to identify significantly enriched pathways. To capture the biological themes that each gene set represented, we compiled their leading-edge subset of genes, and ranked them on the basis of their fold change. For our primary analysis, we compared two groups of subjects dichotomized by a clear bimodal distribution of ventilator-free days (VFDs): the high-VFD subjects had at least 18 VFDs (n = 14) whereas the low-VFD subjects had no more than 7 VFDs (n = 12). GSEA was applied separately in each cell type to patient groups dichotomized by outcome.

Results

Demographic and clinical characteristics of all 26 subjects who had AMs and PBMs collected at study entry are shown in Table 1. Manual inspection of Cytospin slides after negative selection revealed an average purity of 97% for AMs and PBMs.

We first performed principal component analysis using the entire microarray data set across the 26 patients with ARDS with both AM and PBM data and observed that the principal driver of gene expression variability was the large-scale differences between cell-specific transcriptomes (Figure 1). We then compared differential gene expression between AMs and PBMs, and found marked cell-specific differences, with 6,099 differentially expressed genes identified between the two cell types (false discovery rate < 0.01).

Figure 1. Transcriptional landscape of alveolar macrophages (AMs) and peripheral blood monocytes (PBMs) in acute respiratory distress syndrome. Principal component analysis of the AM and PBM transcriptomes in 26 patients at the onset of acute respiratory distress syndrome demonstrated clear segregation between these two cell types, implying that the primary driver of gene expression variability is due to cell-specific differences (AMs, purple spheres; PBMs, green spheres).

Table 2. Enriched Gene Sets Based on Cell Type and Clinical Outcome

VFD ≥ 18 (Good Outcome)				VFD ≤ 7 (Poor Outcome)			
Gene Set	FDR	Gene	FC	Gene Set	FDR	Gene	FC
Alveolar macrophages							
TNF α signaling via NF- κ B	<0.01	<i>CCR7</i>	3.1	MYC targets V1	<0.01	<i>CAV1</i>	2.5
IFN- γ response	<0.01	<i>FOSB</i>	2.6	Peroxisome	<0.01	<i>FHL2</i>	1.8
Inflammatory response	<0.01	<i>DUSP2</i>	2.5	UV response down	<0.01	<i>TFPI</i>	1.6
IFN- α response	<0.01	<i>IL6</i>	2.3	Protein secretion	0.02	<i>SLC25A4</i>	1.6
Allograft rejection	<0.01	<i>STAT4</i>	2.3	Pancreas β cells	0.03	<i>LAMC1</i>	1.6
Hypoxia	<0.01	<i>NR4A2</i>	2.1			<i>ERBB2</i>	1.6
Complement	<0.01	<i>IL23A</i>	2.1			<i>EFEMP1</i>	1.5
IL2 STAT5 signaling	0.01	<i>SLAMF1</i>	2.1			<i>SCGB1A1</i>	1.4
IL6 JAK STAT3 signaling	0.02	<i>CXCL2</i>	2.0			<i>FBLN5</i>	1.4
UV response up	0.03	<i>DLL1</i>	2.0			<i>TSPAN8</i>	1.4
Coagulation	0.03	<i>DNAJB1</i>	2.0			<i>SLC7A1</i>	1.4
Apoptosis	0.04	<i>MMP9</i>	1.9			<i>FOXA2</i>	1.4
Notch signaling	0.04	<i>TNFAIP3</i>	1.9			<i>GJA1</i>	1.3
		<i>MMP12</i>	1.9			<i>IDH1</i>	1.3
		<i>C8B</i>	1.8			<i>ERH</i>	1.3
		<i>GCH1</i>	1.8			<i>ATP2B4</i>	1.2
		<i>HAS1</i>	1.8			<i>ICA1</i>	1.2
		<i>IRAK2</i>	1.7			<i>NFIB</i>	1.2
		<i>CD274</i>	1.7			<i>GNPAT</i>	1.2
		<i>ICOS</i>	1.7			<i>CAV2</i>	1.2
		<i>IL1B</i>	1.7			<i>YWHAQ</i>	1.2
		<i>PROK2</i>	1.6			<i>IARS</i>	1.2
		<i>ZC3H12A</i>	1.6			<i>MAFB</i>	1.2
		<i>HLA-DQA1</i>	1.6			<i>RPS2</i>	1.2
		<i>CTSZ</i>	1.6			<i>SMARCC1</i>	1.2
		<i>IL18BP</i>	1.5			<i>ACP1</i>	1.2
		<i>IL1A</i>	1.5			<i>PTPRM</i>	1.2
		<i>CXCL9</i>	1.5			<i>APEX1</i>	1.2
		<i>CXCR7</i>	1.5			<i>CNBP</i>	1.1
		<i>EIF5A</i>	1.4			<i>C1QBP</i>	1.1
Peripheral blood monocytes							
IFN- α response	<0.01	<i>HLA-DQA1</i>	1.9	MYC targets V1	<0.01	<i>EGR2</i>	2.1
IFN- γ response	<0.01	<i>XAF1</i>	1.3	Oxidative phosphorylation	<0.01	<i>FOSB</i>	1.8
Unfolded protein response	0.01	<i>WFS1</i>	1.3	MTORC1 signaling	0.01	<i>IL1R2</i>	1.6
		<i>ISG15</i>	1.3	Adipogenesis	0.01	<i>EGR1</i>	1.6
		<i>HLA-DMA</i>	1.3	Fatty acid metabolism	0.01	<i>CA2</i>	1.5
		<i>DNAJA4</i>	1.2	IL2 STAT5 signaling	0.03	<i>CXCL10</i>	1.4
		<i>DHX58</i>	1.2	Peroxisome	0.03	<i>AKR1C3</i>	1.4
		<i>SDAD1</i>	1.2	Xenobiotic metabolism	0.04	<i>PTGS2</i>	1.4
		<i>PIM1</i>	1.2	TNF α signaling via NF- κ B	0.05	<i>SH3BGR2</i>	1.3
		<i>TRIM25</i>	1.2	E2F targets	0.05	<i>TUBA4A</i>	1.3
		<i>SLC25A28</i>	1.2			<i>TPST1</i>	1.3
		<i>SAMD9L</i>	1.2			<i>ATP1B3</i>	1.3
		<i>CD86</i>	1.2			<i>FH</i>	1.3
		<i>CD74</i>	1.2			<i>ACTR3</i>	1.3
		<i>DGP2</i>	1.2			<i>PTGES3</i>	1.3
		<i>SPCS3</i>	1.2			<i>ME1</i>	1.3
		<i>SP110</i>	1.2			<i>ID2</i>	1.3
		<i>PARP12</i>	1.2			<i>CNBP</i>	1.3
		<i>PELI1</i>	1.2			<i>PROS1</i>	1.3
		<i>SELL</i>	1.1			<i>HMGB2</i>	1.2
		<i>DNAJB9</i>	1.1			<i>GPR65</i>	1.2
		<i>TNFAIP3</i>	1.1			<i>GSTZ1</i>	1.2
		<i>ADAR</i>	1.1			<i>HSD17B11</i>	1.2
		<i>CIITA</i>	1.1			<i>PPP1R15A</i>	1.2
		<i>MOV10</i>	1.1			<i>SDPR</i>	1.2
		<i>EDC4</i>	1.1			<i>ACOX2</i>	1.2
		<i>PDE4B</i>	1.1			<i>CES1</i>	1.2
		<i>ARFGAP1</i>	1.1			<i>PSMD14</i>	1.2
		<i>ATF3</i>	1.1			<i>ALDH2</i>	1.2
		<i>RBCK1</i>	1.1			<i>ARG2</i>	1.2

Definition of abbreviations: FC = fold change; FDR = false discovery rate; UV = ultraviolet; VFD = ventilator-free days.

Gene sets are enriched hallmark gene sets curated from the Molecular Signature Database (<http://software.broadinstitute.org/gsea/msigdb>). Displayed are the significantly enriched gene sets (FDR < 0.05) for each cell type and outcome and a list of the 30 most upregulated genes (fold change) compiled from all the leading-edge genes (9) driving each of the enriched gene sets. These gene lists represent the predominant biological themes that drove each of the gene set associations listed; however, no individual gene met the threshold for statistical significance after multiple hypothesis testing.

After determining that the cell-specific transcriptional profiles between AMs and PBMs were different in early ARDS, we sought to characterize which biological programs were activated by ARDS in each cell type and associate these programs with patient outcomes. In high-VFD subjects (good outcome), there was clear enrichment of immune and proinflammatory gene sets in AMs, whereas there was a much less diverse immune signature in PBMs isolated from those same patients (Table 2). Notably, PBMs collected from low-VFD subjects (poor outcome) were enriched with several of the same immune and inflammatory pathways that were associated with good outcomes in AMs, such as tumor necrosis factor- α and IL-2/Stat5 (signal transducer and activator of transcription 5) signaling. *IL6*, *IL23A*, *CXCL9* (chemokine [C-X-C motif] ligand 9), and *IL1B* were among the most up-regulated genes found in the leading-edge enriched hallmark gene sets derived from AMs collected from high-VFD subjects, reinforcing the nature of the immunoinflammatory transcriptional signal associated with AMs from patients with good clinical outcomes (Table 2). Finally, we observed an even more robust pattern of distinctive pathway enrichment between AMs and PBMs when applying GSEA using the more extensive canonical pathway gene sets from MSigDB.

Discussion

This report provides the first assessment of paired AM and PBM transcriptional activation in human ARDS. We identified highly divergent patterns of gene expression between AMs and PBMs in subjects with ARDS. Furthermore, we found that the initial transcriptome in AMs procured from patients who ultimately had good outcomes (VFD ≥ 18) was enriched in immunoinflammatory gene sets, whereas enrichment of many of the same pathways in PBMs was associated with poor outcomes (VFD ≤ 7). The broad implication of our finding is that strategies for identifying molecular therapeutic targets in ARDS must take into consideration that organ- and cell-specific responses to this syndrome vary profoundly across different compartments.

Another important implication of this work is that extrapolating transcriptional signals from circulating immune cells to approximate responses in airspace immune cells must be done with caution. Almost all previous studies examining the transcriptome in ARDS have used samples collected from the peripheral circulation as opposed to samples directly procured from the lung (1–4). Our results shed some light on the observed discrepancies between reports based on whole blood transcriptional measurements in ARDS versus studies analyzing products collected directly from the alveolar space. For instance, despite the fact that IL-8 protein levels in BAL fluid have been found to be elevated in early ARDS in multiple studies (10, 11), one of the first genome-wide transcriptional analyses using whole blood RNA of patients with ARDS found decreased expression of *IL8* in the acute phase of ARDS compared with the recovery phase (1). Future studies in ARDS that use “omics” approaches should take into consideration that peripheral blood leukocyte gene expression does not accurately reflect the transcriptional patterns of immune cells in the alveolar space. ■

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Systematic Error in Respiratory Impedance Using Commercial Equipment Calibrated according to the Manufacturer's Instructions

To the Editor:

Measuring lung function in subjects who find the forced expiratory maneuvers required for spirometry difficult, such as preschool-aged children and elderly patients with chronic obstruction pulmonary disease (COPD), is challenging. Theoretically, the forced oscillation technique (FOT) should be ideal for these subject groups, as the measurements are made during gentle tidal breathing without requiring any special breathing maneuvers. Indeed, the clinical utility of FOT has been demonstrated in preschool-aged children with wheeze and patients with COPD (1, 2). However, FOT is also considered to be more effective at showing differences between groups of subjects (e.g., wheezy children vs. control subjects) than in detecting abnormal lung function in individual patients (3). The variable breathing pattern typical of young children results in variable contributions by the influence of flow and volume dependence of the respiratory system to the measurements of respiratory impedance (Zrs) obtained.

The usually low, static reference impedance used for calibration of the commercial FOT devices is of concern, as is the simplistic correction for the impedance of the filter-mouthpiece assembly (FMA) that is likely to introduce errors into measurements of Zrs. These effects would be anticipated to be magnified when measuring

subjects with higher values of Zrs, including young children and patients with COPD.

We have previously published normative data for Zrs for children aged 2 to 13 years and the response to bronchodilator using the commercially available i2M forced oscillation device (Ches Medical; marketed in Italy by Cosmed) (4, 5). We calibrated according to the manufacturer's instructions using a standard impedance of $2 \text{ hPa} \cdot \text{s} \cdot \text{L}^{-1}$ and FMA correction by averaging and storing 10 sets of FMA impedance. The proprietary software then corrects patient Zrs for the FMA using an undisclosed procedure. Given that Zrs of preschool-aged children is substantially higher than $2 \text{ hPa} \cdot \text{s} \cdot \text{L}^{-1}$, we sought to determine whether calibrating according to the manufacturer's instructions would introduce errors into the measurements of Zrs and its resistance (Rrs) and reactance (Xrs) components at the commonly reported frequencies: 6 Hz (R6, X6), 8 Hz (R8, X8), and 10 Hz (R10, X10).

To undertake this study, we used a gold-standard wave-tube assembly as well as the i2M to measure FOT. The wave-tube is more accurate and considered to be the gold standard, as the tube impedance is calculated with precision from the physical characteristics of the tube and the subject is measured as a load-impedance on the tube (1, 6). Regular calibration is accomplished by the adjustment of the gains of the inlet and outlet pressure recording channels (which are potentially time variant) at the start of every measurement session. The wave-tube principle and this pressure gain calibration routine ensure the accurate measurement of mechanical impedances as high as $100 \text{ hPa} \cdot \text{s} \cdot \text{L}^{-1}$. FMA impedance can be calculated and measured precisely, considered as an arrangement of series and parallel impedances, and removed from the subject Zrs (7). The present study was undertaken to determine 1) whether differences between Zrs would be seen when the same subject was measured using each device (bias), and 2) whether systematic bias related to the magnitude of Zrs was present (heteroscedasticity).

We measured Zrs using the i2M and wave-tube assembly in 42 subjects, aged 3.6 to 58.6 years, with Rrs ranging from 2.31 to $13.10 \text{ hPa} \cdot \text{s} \cdot \text{L}^{-1}$. The device order was randomized. The i2M systematically underestimated Rrs and overestimated Xrs (more negative), as shown in Table 1. Heteroscedasticity was also seen in Xrs, with the magnitude of the difference between the two techniques related to the magnitude of the mean Xrs (Figure 1).

Table 1. Bias and Heteroscedasticity in Respiratory Resistance (R6, R8, R10) and Reactance (X6, X8, X10) when Comparing Data Measured with the i2M and the Gold-Standard Wave Tube

	R6	R8	R10	X6	X8	X10
Bias, $\text{hPa} \cdot \text{s} \cdot \text{L}^{-1}$, mean $\pm$ SD	0.27 ± 1.06	-0.02 ± 1.13	0.18 ± 0.92	0.66 ± 1.13	0.53 ± 0.69	0.52 ± 0.71
Heteroscedasticity*						
Coefficient	0.02	-0.03	0.015	-0.49	-0.44	-0.48
r^2	0.003	0.007	0.002	0.28	0.49	0.54
P value	0.74	0.59	0.79	0.0003	<0.0001	<0.0001

*Heteroscedasticity was determined by linear regression: difference = $a + b \cdot \text{average}$, where b is the regression coefficient. r^2 is the square of the Pearson regression coefficient.