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Graphical abstract

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Single Cell RNA Sequencing Identifies an Early Monocyte Gene Signature in Acute Respiratory Distress Syndrome

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ABSTRACT

The acute respiratory distress syndrome (ARDS) results from overwhelming pulmonary inflammation. Prior bulk RNA sequencing provided limited insights into ARDS pathogenesis. We used single cell RNA sequencing to probe ARDS at a higher resolution. Peripheral blood mononuclear cells of patients with pneumonia and sepsis with early ARDS were compared to that of sepsis patients who did not develop ARDS. Monocyte clusters from ARDS patients revealed multiple distinguishing characteristics in comparison to monocytes from patients without ARDS including down-regulation of SOCS3 expression accompanied by a pro-inflammatory signature with up-regulation of multiple type I IFN-induced genes, especially in CD16+ cells. To generate an ARDS risk score, we identified up-regulation of 29 genes in the monocytes of these patients, and 17 showed a similar profile in cells of patients in independent cohorts. Monocytes had increased expression of RAB11A, known to inhibit neutrophil efferocytosis, ATP2B1, a calcium pump that exports Ca2+ implicated in endothelial barrier disruption, and SPARC, associated with processing of pro-collagen to collagen. These data show that monocytes of ARDS patients up-regulate expression of genes not just restricted to those associated with inflammation. Together, our findings identify molecules that are likely involved in ARDS pathogenesis that may inform biomarker and therapeutic development.
INTRODUCTION

The acute respiratory distress syndrome (ARDS) is a clinical syndrome that is often life threatening and affects 3 million people worldwide accounting for up to 10% of intensive care unit (ICU) admissions (1). Its clinical manifestations include acute hypoxemia, noncardiogenic pulmonary edema and reduced lung compliance (increase in stiffness). ARDS can precipitate as a result of a variety of pulmonary insults (pneumonia typically resulting from bacterial or viral infections, gastric aspiration) or non-pulmonary insults (sepsis, trauma, pancreatitis). ARDS carries high mortality (30-40%) and survivors experience long-term sequelae of the syndrome including pulmonary function limitations and sustained neurocognitive deficiencies (2).

Despite decades of intense research and multiple clinical trials, treatment options remain limited relying on supportive care with invasive mechanical ventilation and efforts to reverse the underlying etiology (3–5). Targeted interventions require a deeper understanding of the mechanisms that induce ARDS. Furthermore, ARDS develops rapidly after the initial insult, and since it is not possible to assess lung injury directly, and no consensus is yet reached regarding biomarkers that can be included in the definition of ARDS, indirect clinical criteria are relied upon for a diagnosis of ARDS (6).

During the initial exudative phase of lung injury in ARDS patients, innate immune cells, including neutrophils and monocytes, are recruited to the alveolar airspaces causing damage to epithelial and endothelial barriers resulting in accumulation of proteinaceous edema fluid in the alveoli and interstitium (6–9). During lung injury, resident alveolar macrophages secrete pro-inflammatory cytokines that aid in the recruitment of circulating neutrophils and mononuclear cells to the lung causing sustenance of inflammation and tissue injury (10). The inflammatory phase of ARDS is followed by a fibroproliferative phase characterized by
resolution of inflammation and deposition of collagen (7, 11, 12). Although the inflammatory cascade resulting in acute lung injury is relatively understood in mouse models, mechanistic understanding of ARDS in humans remains limited.

Prior studies have used the microarray platform to compare patients with sepsis to those with sepsis and acute lung injury/ARDS to identify transcripts associated with ARDS. In one study, an eight gene signature was able to distinguish between patients with sepsis and sepsis with acute lung injury (13). Further investigation found elevated RNA of IL-1 superfamily cytokines with a statistically significant increase in IL-1β and a trend towards an increase in IL-18 when comparing patients with ARDS and sepsis compared to patients lacking ARDS (14). IL-18 has been associated with increased mortality in sepsis-induced ARDS (15). Expression of genes related to neutrophil function was also found to be elevated in patients with ARDS when compared to patients with sepsis alone (16). Taken together, these studies suggest that differential gene expression in PBMCs may be associated with ARDS development and underlie mechanisms of inflammatory injury to the lungs in patients with sepsis. However, microarray data are limited in their ability to resolve activated transcription programs at the cellular level. Single cell RNA sequencing (scRNA-seq) overcomes this limitation by unbiased sequencing of transcripts in individual cells and has the potential to provide deeper insights into the mechanisms of ARDS (17).

In this study, we performed scRNA-seq to identify early transcriptomic signatures associated with the development of ARDS in patients with pneumonia-associated direct lung injury. PBMCs were isolated from immunocompetent patients within 24 hours of intubation to test the hypothesis that early transcriptomic changes are identifiable by scRNA-seq that can differentiate between patients who develop ARDS versus those who do not secondary to pneumonia. In our
attempt to identify molecular signatures that are associated with diffuse alveolar injury and capillary leak, ARDS patients selected for this analysis were compared to patients requiring mechanical ventilatory support for sepsis and pneumonia but did not meet clinical criteria for ARDS (18). Our study has identified previously unrecognized differences in gene expression in NK cells and monocyte clusters of patients with ARDS as compared to those who did not develop ARDS that may help in future biomarker and therapeutic development.
RESULTS

Subject characteristics

Peripheral blood samples were collected from four patients at risk for ARDS with pneumonia and sepsis (sepsis only) and three patients who had advanced to ARDS with sepsis and pneumonia (sepsis+ARDS) within 24 hours of initiation of mechanical ventilation (Figure 1A). Subjects ranged in age from 36 to 78 years with median age of 51 years, as summarized in Table 1. A diagnosis of ARDS was made retrospectively according to the Berlin definition of exposure to a known ARDS risk factor within one week and development of bilateral opacities on chest radiograph that are not fully explained by cardiogenic pulmonary edema (18).

Compared to subjects with ARDS, the sepsis only group had a higher proportion of males. One ARDS patient tested positive for influenza but the pathogen was not identified in the other two cases. Ventilator settings at the time of enrollment are included (Table 1).

Cluster identification

Cell types were identified in an unbiased manner after batch correction and normalization. Main clusters commonly observed in PBMCs as well as subpopulations within monocytes and T cells were identified (Figure 1, B and C, Supplemental Tables 1, 2, Supplemental Figure 1, Supplemental Figure 2, A and B). The Mono/B cluster was suspected to be composed of multiplets of monocytes and B cells, while the Mono/T cluster with relatively high proportion of mitochondrial genes indicated poor quality (Supplemental Figure 1). CD14+ monocytes were further clustered into four sub-populations. Two cell clusters could not be identified and were excluded from further analysis. We found a significant decrease in the number of NK cells, which may be due to recruitment of these clusters to the focus of inflammation or differential expression of specific genes, as discussed below. The proportion of B cells was elevated in ARDS (Figure 1D). Genes used to identify cell clusters were conserved in both patient groups (Figure 1E).
Differentially expressed genes in cells from sepsis only versus ARDS subjects

We identified 53 differentially expressed genes between sepsis only and sepsis+ARDS subjects in all cells (Bonferroni-adjusted P value < 0.01, absolute value of average log fold change (logFC) > 0.1, Supplemental Table 3). Among them, several interferon-related genes were up-regulated in sepsis+ARDS compared to sepsis only patients. In a recently reported study that employed bulk-RNA sequencing, one of these genes, IFI44L, an important paralog of the gene Interferon Induced Protein 44, an interferon stimulated gene (ISG), was found to be differentially expressed between ARDS and ARDS-hematopoietic stem cell transplanted patients (19). Other intriguing genes included HLA-DQB1, a member of the HLA complex, and NAMPT, a regulator of intracellular NAD pool and cellular metabolism (20). AREG, which encodes amphiregulin, was among the down-regulated genes. Down-regulation of AREG induces epithelial cell apoptosis in LPS-induced lung injury in mice (21).

Canonical signaling pathways are activated in NK cells in ARDS

In agreement with a reduced frequency of NK cells shown in Figure 1D, the t-SNE profiles in Figure 2A show reduction in NK cell numbers in ARDS patients. Figure 2B shows that overall gene expression was also reduced in the NK cells, which was especially true for specific genes such as PTGDS (encodes prostaglandin D2 synthase). Interestingly, genetic deletion of the isoform of prostaglandin D2 synthase found in hematopoietic cells resulted in increased inflammation at the outset in a mouse model of peritonitis and also impaired resolution of inflammation (22). Furthermore, prostaglandin D2 has been shown to inhibit NK cell secretion of type 1 cytokines (23). Expression of the interferon-inducible genes, IFI44L and IFITM3, was up-regulated in NK cells of ARDS patients, while, interestingly, expression of IFNGR1 was undetectable (Figure 2C). IFNGR1 is ubiquitously expressed by all cell types and its down-regulation may prevent pro-inflammatory responses to IFN-γ as recently observed in human
monocytes (24). Pathway enrichment analysis using the 102 suppressed genes (Bonferroni-corrected P value < 0.01, log-fold-change < -0.1) revealed pathways inhibited in ARDS patients. Altogether, 29 pathways were found to be significantly down-regulated (Bonferroni-corrected P < 0.01, Supplemental Table 4). The most significantly enriched pathways in each biological process group are shown in Figure 2D. In ARDS patients, the combined down-regulation of genes that negatively regulate NF-κB activation, negatively regulate mitogen-activated protein (MAP) kinases, and also negatively regulate serine/threonine protein kinases suggests an increase in activation status of NK cells in the context of ARDS.

**Type I interferon-associated pathways and transcription factors are up-regulated in the monocytes of ARDS patients**

Monocytes are heterogeneous and dynamic in circulation. We identified four clusters of CD14+ monocytes and one cluster of CD16+ monocytes (Figure 3, A and B). The first cluster of CD14+ monocytes showed high expression of CES1, also known as monocyte esterase. CD74 along with HLA family genes were markers for the second cluster. The third cluster expressed higher levels of CTSD, that encodes lysosomal protease cathepsin D, while the last cluster of monocytes had high expression of platelet basic protein precursor gene (PPBP) (Figure 3C). These four clusters of CD14+ monocytes could represent various stages of differentiation of monocytes, which may have functional consequences in ARDS. The proportion of each of the monocyte subsets in the two patient groups was not significantly different, which is shown in Supplemental Figure 3.

Pathways enriched in the top differentially expressed genes (DEGs) in each monocyte subpopulation in patients with sepsis+ARDS relative to those in patients with sepsis only were examined (Bonferroni-adjusted P value < 0.01, logFC >0.1, Supplemental Table 5). Pathways corresponding to up-regulated genes are illustrated in Figure 3D, where each node denotes a
biological process while colors of the nodes distinguish the five monocyte sub-populations. Both CD16+ monocytes and CD14+ monocytes from the CES1 (cluster 1) and CD74 (cluster 2) clusters showed up-regulation of type I interferon signaling pathways. CD74 (cluster 2) and CTSD (cluster 3) monocytes were linked to neutrophil migration and degranulation, respectively. The CTSD (cluster 3) monocyte cluster was enriched in pathways related to host defense, including responses to lipopolysaccharide and virus. Pathways involved in platelet aggregation were up-regulated in ARDS in the CD74 (cluster 2) monocyte cluster. Analysis of gene regulatory networks in monocytes revealed transcription factors that are differentially activated in monocytes of ARDS versus sepsis-only patients (Figure 3E). The interferon regulatory transcription factor (IRF) family proteins IRF1, IRF2 and IRF7 and Signal Transducer and Activator of Transcription (STAT) family (STAT1, 2) were activated in the monocyte populations of ARDS patients, while CEBP/β, FOXO1 and ATF4 were suppressed in these cells. By RNA-Seq, we previously showed that human alveolar macrophages exhibit a strong type I IFN gene signature and increased IRF7 expression when stimulated by LPS ex vivo (25).

**SOCS3 expression is down-regulated in the monocytes of ARDS patients**

We next examined whether increased expression of IFN-induced genes in the ARDS group was caused by a deficiency of negative regulators of JAK-STAT signaling such as SOCS3. As published previously, SOCS3 efficiently blocks type II IFN (IFN-γ)-induced responses in cells stimulated by IL-6 (26). Deletion of SOCS3 in macrophages was shown to induce aberrant STAT1 activation in response to IL-6 (27). In subsequent studies, SOCS3 was also shown to inhibit type I IFN (IFN-β)-induced signaling in macrophages (28). Given that serum IL-6 and IFN-γ levels have been associated with poor prognosis in ARDS (29, 30), we examined whether the monocyte populations in the ARDS patients had a defect in SOCS3 expression as compared to cells from patients with sepsis only. This analysis showed that indeed SOCS3 expression was
barely detectable in all of the monocyte clusters in ARDS patients while robust expression was detected in cells from patients with sepsis only (Figure 4). Since SOCS1 also inhibits type I (31) and type II (32) IFN signaling, we also examined its expression in monocytes (Supplemental Figure 4A). SOCS1 expression, however, was low in all CD14 and CD16-expressing monocyte clusters. We next examined IFN receptor expression and whether genes that are known to be induced by IFN-γ or type I IFNs are differentially expressed in the cells from both groups of patients. While all monocyte clusters were found to express IFNGR1 at similar levels, expression of IFNAR1 was low (Supplemental Figure 4). Low expression of IFNAR1 may be due to ligand-mediated down-regulation of the receptor, as was recently reported for IFNGR1 in human monocytes (24). Expression of GBP1, which encodes a member of the family of guanylate-binding proteins (GBPs), with functions in host defense (33, 34), was expressed at a higher level in CD16+ monocytes and the expression of multiple ISGs was also higher in the cells of ARDS patients (Figure 4). Treatment of human monocytes with IFN-β was previously shown to upregulate genes such as GBP1, IFITs and IFI44L (35). Some of these IFN-target genes (GBP1, IFITM1) were found to be preferentially upregulated in CD16+ monocytes. However, the expression of GABARAP, which is an IFN-γ-activated gene and shown to be important for cellular distribution of GBPs (36) and anti-microbial responses (36), or of LYN, another IFN-γ-induced gene (37); was also similar in the cells from both groups (Figure 4). Taken together, these data identify a molecular defect in the expression of SOCS3 in circulating monocytes of ARDS patients, which our data suggest has downstream consequences that includes up-regulation of multiple genes, primarily targets of type I IFNs.
Differential monocyte gene expression identifies a predictive score to identify ARDS from bulk transcriptomic data

The data shown above identified a preferential up-regulation of type I IFN-regulated genes in the CD16+ monocyte cluster, which comprises ~10% of all monocyte populations in the peripheral circulation of humans. We next asked whether it was possible to identify a core gene signature set that distinguished the transcript profile of the total monocyte population in ARDS patients as compared to that in patients with sepsis only but at risk for ARDS. We identified differentially expressed genes (DEGs) in monocytes in our dataset as candidates for generating an ARDS risk score (see methods, Figure 5A, Supplemental Table 6). The weight of each gene was calculated as logFC. In our scRNA-seq data, we identified 29 genes that were selectively differentially regulated in the monocytes of ARDS patients as compared to that in sepsis only patients, with Bonferroni-corrected P values < 0.01 in both Wilcoxon rank number test and MAST (38) and with logFC greater than 0.13 (Figure 5A). One of these genes, RAB11A, is a small GTPase that inhibits neutrophil efferocytosis, and targeting this gene in macrophages was shown to promote resolution of LPS-induced lung injury (39). Increased expression of RAB11A in cells of ARDS patients, therefore, can be expected to result in increased accumulation of neutrophils in the peripheral blood with potential for increased pulmonary infiltration. Related to this possibility, we also identified increased expression of the gene ATP2B1, encoding a calcium pump (40), in the monocytes of ARDS patients, the functional correlate of which would be increased cellular export of calcium with the potential to impact neighboring cells including endothelial cells. Increased calcium signaling in endothelial cells can cause endothelial barrier disruption, which is a feature of ARDS (41). Additional genes of interest that were expressed at a higher level in the cells of ARDS patients included SPARC, which promotes processing of pro-collagen to collagen (42), lung fibrosis being a feature of ARDS. Our data suggest that both fibroproliferation and monocyte-expressed SPARC may contribute to increase in fibrosis in ARDS patients. SPARC was recently identified as a marker
of myeloid derived suppressor cells (MDSCs) (43), and interestingly, we detected increased expression of another gene, *NRGN* that has been also found to be expressed by MDSCs (Gabrilovich Condamine US patent 2018). MDSC expansion has been associated with nosocomial infections in patients with sepsis (44). Increased expression of *PDK4* in cells of the ARDS group may contribute to increased lactate levels in these patients, which has been associated with increased risk for mortality (45).

We next assessed the relative expression of these 29 genes in peripheral blood monocytes of sepsis only versus sepsis+ARDS patients using available datasets in the Gene Expression Omnibus (GEO) database. The relevant available human datasets are summarized in Supplemental Table 7. After combining these datasets, 8 sepsis patients with infection by Gram-negative bacteria, and 26 ARDS patients were included in the initial analysis. The datasets underwent individual-wise normalization and standardization across genes. Among the 15,555 genes whose expression was available in both datasets, 17 up-regulated genes among the 29 differentially expressed in the monocytes of ARDS patients in our data were also up-regulated in the cells of ARDS patients in the bulk RNA expression datasets (Figure 5A, Supplemental Table 6). A significant difference was observed between scores of the septic group versus the ARDS group (P < 2.2e-16, Figure 5B). No significant difference in expression was evident between scores when genes were randomly selected from the groups in a sensitivity analysis (P value = 0.600, Figure 5C). Of note, due to the high stringency set for this analysis with the application of both Wilcoxon Rank Sum Test and MAST, and also a cutoff of fold change to 0.1, *SOCS3* and interferon-stimulated genes identified as being differentially expressed in the monocytes between the two groups using MAST alone (Figure 4), a method tailored for analysis of scRNA-seq data, were precluded in this 29 gene set. The schematic in Figure 6 depicts potential mechanisms by which the differentially expressed genes identified in this analysis might impact ARDS.
DISCUSSION

ARDS is a lethal clinical syndrome that occurs commonly in the ICU although the pathogenesis in humans is poorly understood. We hypothesized that utilization of single cell transcriptomics early in the disease course of ARDS would lead to the identification of novel molecular signatures that characterize ARDS. To the best of our knowledge, our study is the first to apply scRNA-seq technology to a cohort of patients with pneumonia and sepsis who have advanced to ARDS and those with pneumonia and sepsis only but at risk for ARDS and identify distinct molecular profiles in their peripheral blood immune cells. In a prior study of this cohort comparing patients with a known risk factor for ARDS but not meeting clinical Berlin criteria for the diagnosis of ARDS (18) to patients who developed ARDS, the two groups were indistinguishable by clinical and biomarker parameters (46). The present study shows that ARDS and patients at risk for ARDS can be distinguished at the cellular and molecular level using the finer resolution afforded by scRNA-seq technology. The results of our analysis could form the basis for biomarker discovery aimed at distinguishing ARDS from respiratory complications without ARDS.

Our data advances prior bulk RNA sequencing analyses comparing sepsis only to sepsis with ARDS by being able to distinguish between cellular composition and gene expression in cells from the two groups. At the cellular abundance level, we have identified a reduced frequency of peripheral blood NK cells in ARDS patients as compared to that in the other group. However, despite reduced frequency, the NK cells in the ARDS group appeared to have a higher activation profile based on marked down-regulation of IFNGR1 expression and also of multiple negative regulatory pathways that determine activation of transcription factors such as NF-κB and of MAP kinases that promote inflammation. In our analysis, increased expression of IFITM3 in NK cells of ARDS patients, which is primarily induced by type I IFNs as an antiviral factor (47),
and which inhibits cell proliferation (48) may explain the reduced frequency of the cells in this group. Also, down-regulation of IFNGR1 suggests an inability to regulate inflammatory pathways induced by IFN-γ, as recently demonstrated for human monocytes (24).

One distinct feature of cells from ARDS patients that we have identified is a heightened response to IFNs. The overall analysis of DEGs in our study suggests differential up-regulation of Type I IFN-induced pathways that include expression of transcripts for GBP family members, ISGs and HLA. Increased expression of HLA-DQb1 was previously described in an ARDS cohort suggesting increased functional activation of monocytes as antigen-presenting cells (49, 50). Circulating monocytes can also differentiate into DCs when recruited to the lung (50).

Whole blood microarray studies have previously identified neutrophil-related genes to be differentially regulated in sepsis-associated ARDS (16), and pathway analysis identified ‘viral infection’ to be notably up-regulated in ARDS. Furthermore, neutrophil ISG over- and under-expression is associated with reduced survival in ARDS (51). Our data demonstrating activation of Type I IFN-induced pathways supports the importance of exaggerated Type I IFN response in the development and outcome of ARDS. Of note, tonic type I IFN is known to be important for immune homeostasis (52) but unregulated signaling downstream may be detrimental as suggested by our data. Along these lines, we show deficient SOCS3 expression in the peripheral blood monocytes of ARDS patients. SOCS3 negatively regulates signaling downstream of IL-6 and also type I and type II IFNs. Interestingly, SOCS3 deficiency causes IL-6 to induce an IFN-γ-like response in monocytes via STAT1 activation (26). Our findings are further supported by the finding that SOCS3 deficiency in Ly6C+ myeloid cells enhances experimental LPS-induced acute lung injury (53). Given that the genes GBP1, IFI44L, IFITM1 and ISG20, found to be differentially up-regulated in the monocytes of ARDS patients, particularly in the CD16+ subset, are known to be type I IFN-inducible (35), it appears that
SOCS3 deficiency in the monocytes of ARDS patients renders them more responsive to circulating levels of type I IFNs than to increased IL-6-mediated IFN-γ-like signaling. Similar expression of GBP2 (54), GABARAP (36) and LYN (55) in the monocytes of the two groups of subjects, genes typically associated with IFN-γ signaling, suggests absence of enhanced response to IFN-γ in the monocytes of ARDS patients. SOCS3 expression has been inversely associated with the expression of multiple miRNAs in different cell types (56–58). In addition, epigenetic modification of the SOCS3 gene involving promoter hypermethylation was also associated with suppression of SOCS3 expression especially in cancer (59, 60). It would be interesting to determine in future studies the molecular mechanisms that underlie low SOCS3 expression in the monocytes of ARDS patients. Taken together, we propose a SOCS3\text{low} signature in peripheral blood monocytes as a biomarker of ARDS.

We identified four clusters of CD14+ monocytes and one cluster of CD16+ monocytes in our patient cohort. The frequency of each monocyte cluster was not significantly different in ARDS. A third intermediate CD14+CD16+ monocyte subset that has been described in the peripheral blood of humans was not detected in our analysis. Monocytes are crucial regulators of immunity (61). Peripheral blood monocytes have been validated to play an important role in lipopolysaccharide (LPS)-induced lung injury in experimental models. Along with resident alveolar macrophages and recruited alveolar monocytes, they may direct neutrophil-mediated lung injury that characterizes ARDS development (62, 63). A prior study associated recruited monocytes/immature macrophages in the BAL fluid of sepsis patients with ARDS with decreased survival (9). As presented in our data, increased expression of specific genes in peripheral blood monocytes of ARDS patients may play an important role in multiple aspects of ARDS pathogenesis including endothelial cell leak and increased neutrophil recruitment to the lungs. In addition to identifying a heightened type I IFN response, preferentially in the CD16+
subset, we detected up-regulation of platelet aggregation pathways in the CD74^{hi}CD14+ monocyte cluster 2. Platelets are believed to play a role in ARDS pathogenesis through their interaction with the immune system (64).

Monocytes from sepsis+ARDS patients as a whole displayed differential expression of 29 genes as compared to that in monocytes from the sepsis only group. This differential expression profile may have important functional ramifications in ARDS (Figure 6). One such gene upregulated in the cells of the ARDS group is PDK4 which inhibits pyruvate dehydrogenase (65) thereby limiting entry of pyruvate into the TCA cycle and causing increased production of lactate. Increased PDK4 expression in the monocytes of ARDS patients may underlie increased pulmonary and blood lactate levels in ARDS patients (45, 66). We also detected higher expression of ATP2B1 in the monocytes of ARDS patients. ATP2B1 is a calcium transporter that was shown to couple ATP hydrolysis with the transport of calcium from the cytoplasm to the extracellular space in endothelial cells thereby maintaining intracellular calcium homeostasis (40). Increased Ca2+ signaling has been implicated in pro-inflammatory responses and barrier disruption in endothelial cells in ARDS (41) in which monocyte-expressed ATP2B1 may play an important role. Our data also show increased expression of RAB11A that encodes a central recycling endosome component (67), in the monocytes from ARDS patients. The same was true for RAB11FIP1 (RAB family interacting protein 1), a key effector molecule for RAB11A. RAB11A promotes cycling of the metalloproteinase ADAM17 from the cytoplasm to the cell surface, which causes proteolytic cleavage of CD36 from the cell surface. CD36 facilitates phagocytosis of apoptotic neutrophils and it has been shown that RAB11A tonically inhibits macrophage phagocytic functions important for removal of apoptotic neutrophils (39). Interestingly, 17 of these genes in the 29 gene set differentially expressed in the monocytes of ARDS patients in our dataset were also found to be expressed at a higher level in the PBMCs of ARDS patients in independent patient cohorts. Collectively, the gene expression profile shows increased
expression of RAB11A in monocytes implicated in defective clearance of neutrophils and increase in gene expression linked to vascular leak in ARDS.

Although these data identify potential novel mechanisms that underlie ARDS pathogenesis, there are limitations. While the small sample size increases the opportunity for influence of confounding factors due to the clinical characteristics of the patients and treatments that cannot be adequately controlled, prior studies utilizing this technology have drawn biologically relevant conclusions from similar patient numbers (68, 69). In addition, we validated differentially expressed genes in our study across publicly-available bulk RNA datasets generated from microarray analysis of RNA isolated from monocytes of patients with sepsis only and with sepsis and ARDS. Despite these limitations, the striking ability to differentiate between sepsis and ARDS using a monocyte gene signature derived from our single cell data highlights the importance of studying peripheral blood monocytes in ARDS, which could be useful for the generation of a diagnostic biomarker panel for individuals at risk for developing ARDS. While caution must be used when comparing transcriptomic profiles of circulating monocytes and alveolar macrophages to extrapolate mechanisms of pathogenesis (70), results of a prior study has suggested the role of recruited monocytes in poor survival of ARDS patients (9). Also, our ability to clearly differentiate between sepsis and ARDS using peripheral blood samples has strengths in ease of sample accessibility. Future studies assessing single cell transcriptomics of alveolar cell populations are clearly warranted given prior demonstration of the prognostic utility of studying alveolar myeloid populations (71, 72).

Overall, our findings achieve a cell-specific resolution of transcriptomics not previously attempted in ARDS that enrich the mechanistic understanding of genes associated with ARDS risk and mortality (73). We identify increased type I IFN signaling in ARDS that may be caused by down-regulation of SOCS3 expression in the cells of ARDS patients that triggers a pro-
inflammatory phenotype facilitated by increased activation of IRF and STAT transcription factors. We highlight a SOCS3\textsuperscript{low} phenotype of monocytes as a potential biomarker of ARDS. In addition, we show elevated expression of multiple genes in the monocytes of ARDS patients that together may play important roles in precipitating ARDS. In addition, we show reduced frequency of NK cells and complete lack of \textit{IFNGR1} expression as additional features of ARDS. These data add to observations in prior studies identifying a single nucleotide polymorphism in mitogen-activated protein kinase kinase kinase 1 (MAP3K) correlated to ventilator-free days in ARDS (74) and plasma angiopoietin-2 as a potential causal biomarker of sepsis-induced ARDS (75). While only patients with pneumonia as an ARDS risk factor were studied, these findings have important implications in the pathogenesis of ARDS overall as well as in identifying potential pathways to therapeutically modify disease course and reduce the burden of ARDS in the ICU. Future temporal studies of patient samples by single cell transcriptomics that would include patients with various ARDS risk factors would help to more completely characterize the immunopathology of ARDS.
METHODS

All reagents and materials used in this study are listed in Supplemental Table 8.

Study design and sample collection

Patients samples were selected from critically ill mechanically ventilated patients aged >18 years old in the University of Pittsburgh Medical Intensive Care Unit that were prospectively enrolled in the Acute Lung Injury Registry as described previously (46). Blood samples were obtained at the time of enrollment and patients were assigned to either at risk for ARDS with sepsis (sepsis only) and three who had ARDS with sepsis (sepsis+ARDS) meeting the Berlin criteria (18) based on a retrospective review by a consensus committee of critical care physicians. Patients at risk for ARDS with sepsis caused by pneumonia were selected in an attempt to reduce confounding signals from other sources of sepsis. Sepsis was defined as organ dysfunction manifested by the need for mechanical ventilation in the setting of a dysregulated immune response to presumed or proven infection (pneumonia) (76). Blood samples collected >24 hours from the time of intubation were excluded. Patients were excluded if they were recipients of a transplanted organ, had active malignancy, or received systemic immunosuppression or systemic glucocorticoids.

PBMC isolation and cryopreservation

Peripheral blood was drawn into a tube with acid citrate dextrose (ACD) then transferred to a 50ml Falcon tube. Blood was diluted approximately 1:1 with room temperature sterile phosphate-buffered saline (PBS)-LPS tested. After layering Ficoll Paque PLUS, the samples were centrifuged at 450g for 30 minutes at 25°C in a swinging-bucket rotor without braking. The mononuclear cell layer at the interphase was transferred and washed 3 times with 15ml PBS. PBMC were resuspended in cold freeze medium containing 10% dimethyl sulfoxide, 40% fetal bovine serum and 50% X-VIVO15 medium and slowly cooled to -80°C in a Nalgene Cryo 1°C freezing container. Samples were stored in liquid nitrogen. PBMCs were thawed slowly in a
37°C water bath and transferred dropwise to warm cRPMI containing 10% human AB serum and 0.05M 2-mercaptoethanol. PMBCs were spun down at 350xg for 7 minutes at 4°C and gently resuspended in PBS with 0.04% bovine serum albumin (BSA). As previously reported (77), our pilot studies comparing fresh and cryopreserved cells from healthy controls closely matched each other in average gene expression and therefore only cryopreserved cells of all patients were used for scRNA-seq in this study.

**Single cell RNA sequencing and quality control**

scRNA-seq was performed using 10X Genomics Single Cell 3’ solution, version 2, according to the manufacturer’s instructions with 7,000 cells loaded for each sample (n=4 sepsis only and n=3 sepsis+ARDS). Libraries were sequenced on the HiSeq Platform (Illumina) from Novogene and raw BCL/fastq files were analyzed using Cell Ranger version 3.0.2 (10X Genomics). Low-quality cells were removed if they expressed <3000 genes/cell and had more than 20% mitochondrial and 10% hemoglobin gene expression. Post-quality control, 24,806 cells with mean reads 50,419/cell remained for downstream bioinformatic analyses.

**Data processing and bioinformatic analysis**

Samples were analyzed using Seurat (version 3.0, https://satijalab.org/seurat/) for batch correction and cell type identification (78). Alignment between samples was achieved by identifying gene–gene correlation patterns that are conserved across data sets through canonical correlation analysis (CCA). The cells were then embedded in a shared low-dimensional space and normalized using nonlinear ‘warping’ algorithms (78). We subsequently identified discrete cell populations by a shared-nearest neighbor (SNN) graph-based clustering method. Differential gene expression analysis was applied in each cell type with two methods as reciprocal validation: Wilcoxon rank number test and MAST (38), the former as the most commonly used and the latter customized for scRNA-seq data. Pathway enrichment analysis
was further conducted with ClueGO (79) based on GO biological process databases (04/09/2018).

**Functional analysis of subpopulations in monocytes**

Differential gene expression analysis was performed in each of the monocyte clusters, and the log-transformed fold changes (log\_2 FC) were used to perform hierarchical clustering between genes. 5 sets of genes were identified and demonstrated different expression patterns between monocytes from sepsis only and sepsis+ARDS patients. Top differentially expressed genes in each sub-cluster tested for enrichment of biological pathways with ClueGO. Gene regulatory networks were analyzed by SCENIC (80) within monocytes.

**Validation in GEO bulk RNA datasets**

An ARDS risk score was generated based on the findings from our scRNA-seq data. Significant differentially expressed genes in monocytes were selected with Bonferroni-corrected P values less than 0.01 in both Wilcoxon rank number test and MAST test and an absolute value of log-transformed fold change greater than 0.1. The weight for each gene was calculated as log fold change divided by negative log\_{10} Bonferroni-corrected P value in MAST test. The available human monocyte datasets on GEO (GSE89953, GSE46955) were normalized to distribution of zero-mean and one-standard-deviation in each individual. Risk score was calculated as W \times E, where W represents the weight of each gene, while E represents the normalized expression matrix. Risk scores are shown in the box plots stratified by disease status. In addition, sensitivity analysis was performed with scores generated using randomly selected gene sets of the same number with the same weights for 10 replicates to reduce the potential bias in the datasets.
Data availability

Raw single cell RNA sequencing data (fastq files) and processed UMI matrices from our scRNA-seq experiment are available on GEO (GSE151263). Two public human RNA microarray datasets can be accessed from GEO (GSE89953, GSE46955).

Statistics

Differential gene expression analysis was conducted between identified clusters to find marker genes for each cell type using Wilcoxon rank sum test. Further differential analysis between sepsis only and sepsis+ARDS patients was applied within each cell type using two methods as reciprocal validation: Wilcoxon rank number test and MAST. Bonferroni-correction was utilized to adjust for multiple testing, and the cutoff P value of 0.01 after adjustment was set for all differential expression analysis. Pathway enrichment analysis was conducted with ClueGO (79), which utilizes the Fisher Exact Test with cutoff as Bonferroni-corrected P < 0.01. GEO dataset gene expression significance in Supplemental Table 6 was performed using a Student’s t test with two tails.

Study approval

Informed consent was obtained from all patients or surrogates enrolled in the Acute Lung Injury Registry. The study was approved by the Institutional Review Board of the University of Pittsburgh (IRB #19050099).
AUTHOR CONTRIBUTIONS

W.C., A.R. and P.R. conceived and designed the study. Y.J., B.R.R., J.C, and S.D. conducted the primary analysis and interpreted data. J.S.L., G.D.K. and B.J.M. participated in data analysis. Y.J. and B.R.R. prepared the first draft of the manuscript. All authors reviewed the draft for intellectual content and approved submission of the final version of the manuscript.

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COMPETING INTERESTS

The authors have declared that no conflict of interest exists.

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**TABLES**

**Table 1. Patient characteristics.** Patient demographics, pneumonia pathogen and ventilator settings at the time of enrollment are shown. SOFA, sequential organ failure assessment; PEEP, positive end-expiratory pressure.

| Sample  | Age | Gender | Sepsis | ARDS | Tidal Volume (cc/kg) | Pneumonia Pathogen                      | SOFA | PEEP | Plateau Pressure |
|---------|-----|--------|--------|------|----------------------|-----------------------------------------|------|------|-----------------|
| Sepsis\_1 | 65  | Female | +      | -    | +                    | Klebsiella pneumonia                     | 6    | 7.09 | 5               |
| Sepsis\_2 | 62  | Male   | +      | -    | +                    | Klebsiella oxytoca, MSSA                 | 7    | 7.28 | 14              |
| Sepsis\_3 | 51  | Male   | +      | -    | +                    | Enterobacter species                    | 6    | 7.16 | 5               |
| Sepsis\_4 | 78  | Male   | +      | -    | +                    | Enterobacter aerogenes, E coli, MRSA    | 9    | 7.1  | 5               |
| ARDS\_1  | 73  | Female | +      | +    | +                    | Influenza                               | 7    | 8.05 | 8               |
| ARDS\_2  | 51  | Female | +      | +    | +                    | N/A                                     | 4    | 7.32 | 8               |
| ARDS\_3  | 36  | Female | +      | +    | +                    | N/A                                     | 4    | 5.84 | 18              |
Figure 1. scRNA-seq analysis reveals the cellular composition of PBMCs in sepsis only and sepsis+ARDS patients. (A) The schematic illustrates the experimental design and workflow. (B) PBMCs were analyzed from sepsis only (n=4) and sepsis+ARDS (n=3) patients and projected with uniform manifold approximation and projection (UMAP) plots with colors and the number in parenthesis indicating the identified cell cluster. (C) The heat map depicts the marker genes corresponding to each cluster identified in (B). (D) The frequency of each cell type is depicted in the columns. Each patient is represented by an individual color in a shade of blue-green (sepsis only) or orange-red (sepsis+ARDS). The yellow dashed line represents an equal
frequency between the two groups since there are 4 sepsis only patients and 3 sepsis+ARDS patients. (E) The dot plot shows identification of marker genes for each cell cluster with the size of the dot corresponding to the percentage of cells within the cell population expressing the gene, and the brightness of the color representing the average expression level across all cells within the cluster. Blue and red dots indicate sepsis only and sepsis+ARDS patients, respectively. Abbreviations: CD14Mono (CD14+ monocyte), CD8 (CD8+ T cell), CD4T (CD4+ T cell), B (B cell), NK (NK cell), NKT (NK T cell), CD16Mono (CD16+ monocytes), Mk (megakaryocyte).
Figure 2. NK cells show a distinct gene expression profile in ARDS patients. (A) NK cells from sepsis only (n=4) and sepsis+ARDS (n=3) patients are plotted in a t-SNE distribution. (B) The heat map shows differential gene expression in NK cells in the two patient groups. The overall log$_2$ fold change of combined patient samples is indicated on the right hand side of the plot. (C) Violin plots of IFNGR1 and IFITM3 in sepsis only and sepsis+ARDS patients. (D)
Significantly down-regulated genes in NK cells in ARDS patients were analyzed by ClueGO with each node representing the identified pathway. The size of the node corresponds to the enrichment significance.
Figure 3. Monocyte clusters are identified in sepsis only and sepsis+ARDS patients. (A) Monocyte clusters in combined patient samples (n=4 sepsis only and n=3 sepsis+ARDS together) are identified by t-SNE distribution. The number in the parenthesis corresponds to the color-coded cell cluster. (B) A heat map of gene expression in the pooled monocyte clusters is
associated with the corresponding cluster in (A) using the color-coded bar at the top. (C) Violin plots demonstrate expression of the genes that identify each monocyte cluster. (D) Enriched biologic pathways for up-regulated genes in sepsis+ARDS patients are demonstrated using ClueGO biological processes. Each node represents a biological process, and the size of the nodes correspond to the enrichment significance. Colors in the nodes correspond to the monocyte clusters in (A). (E) The heat map demonstrates monocyte transcription factor module expression by patient group and monocyte cluster with log₂ fold change illustrated on the right side. Data shown represent results obtained from pooled samples in each group.
Figure 4. Down-regulation of SOCS3 expression in monocyte clusters and increase in expression of interferon-stimulated genes. Violin plots of expression of selected genes in the monocyte clusters are shown for sepsis only (n=4) and sepsis+ARDS (n=3) patients. * signifies
genes significantly differentially expressed between sepsis only and sepsis+ARDS patients within each monocyte population, at Bonferroni-corrected P value < 0.01 in MAST test, but not in Wilcoxon rank sum test; † genes significant at Bonferroni-corrected P value < 0.01 in both MAST and Wilcoxon rank sum test.
Figure 5. Differential gene expression in monocytes can distinguish between sepsis only and sepsis+ARDS patients. (A) Heat map showing expression levels and weights assigned to each of the 29 genes that distinguish between patients with sepsis (n=8) only and those with sepsis + ARDS (n=26) in our scRNA-seq data as compared to microarray gene expression data of peripheral blood monocytes that were publicly available in two datasets. * indicates genes
upregulated in both the scRNA-seq dataset and publicly-available datasets. (B) Comparison between groups using the ARDS risk score comprising 29 genes versus (C) a random selection of genes. Box and whisker plots show the median (bar) with interquartile range (box) and upper/lower limit within 1.5 interquartile ranges from the box range (whiskers). The red dot indicates the mean of the score values. In panel B, values of the box plot for scores of sepsis only patients are: minimum=-0.23, lower=-0.19, middle=-0.18, upper=-0.15, maximum=-0.15. For sepsis+ARDS patients: minimum=-0.11, lower=-0.09, middle=-0.08, upper=-0.07, maximum=-0.05. In panel C, values of the box plot for scores of sepsis only patients are: minimum=-0.29, lower=-0.12, middle=-0.01, upper=0.11, maximum=0.26. For sepsis+ARDS patients: minimum=-0.26, lower=-0.14, middle=-0.04, upper=0.06, maximum=0.31.
Figure 6. Potential immune mechanisms contributing to the development of ARDS. (A) Monocyte subsets (particularly CD16+ monocytes) down-regulate SOCS3 expression, which promotes type I IFN signaling through STAT and IRF pathways. Reduced SOCS3 levels may also modulate IL-6 signaling. (B) NK cells have increased expression of canonical activation signaling cascades including NF-κB, MAPK and IFN-stimulated genes (IFITM3). (C) Monocytes with increased expression of RAB11A may impair neutrophil efferocytosis leading to persistent alveolar inflammation. (D) Increased ATP2B1 is involved in calcium efflux, which could contribute to alveolar capillary leak and the development of non-cardiogenic pulmonary edema.
(E) Increased PDK4 could contribute to lactic acidosis through inhibition of pyruvate dehydrogenase. (F) Monocytes with increased SPARC and NRGN expression could be markers of myeloid-derived suppressor cells (MDSCs). SPARC is involved in collagen deposition, which is observed in the fibro-proliferative stage of ARDS.