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Teaching an Old Intensivist Neutrophil Tricks: Using Alveolar Neutrophilia to Diagnose Ventilator-associated Pneumonia

An insidious belief pervades the modern ICU—that in cases of diagnostic uncertainty, broad-spectrum antibiotic therapy is “safest for the patient.” This idea is likely a vestige of that bygone era before multidrug-resistant bacteria, when faith in the everlasting efficacy of antimicrobials was strong, and responsible doctors protected their patients from infection without regard for pretest probability.

Fortunately, this belief has been vigorously challenged. Antibiotic overuse is now known to be a clear contributor to the spread of drug resistance, which the World Health Organization has declared to be one of the greatest current threats to human health. It has further predicted that in the absence of improved stewardship, we will enter a postantibiotic era by 2050, with an associated 10 million deaths due to multidrug-resistant bacteria per year (1).

If this apocalyptic prediction were not deterrence enough, it is also clear that inappropriate antimicrobial therapy poses an immediate threat to the patient (2). Risks include life-threatening drug reactions such as Stevens–Johnson syndrome and anaphylaxis, as well as more common complications such as

drug–drug interactions and nephrotoxicity. A more recently appreciated hazard is the profound disruption of gut microbiota produced by antibiotics, a condition termed dysbiosis. Although the full consequences of dysbiosis have yet to be elucidated, it is clearly linked to *Clostridioides difficile* colitis, a highly prevalent and often deadly infection (3). Altogether, the adverse effects associated with unnecessary antibiotics have been shown to worsen mortality in a number of studies by independent groups (4, 5).

It is therefore imperative for both the community as a whole and the individual patient that we develop highly sensitive diagnostic tools to rule out bacterial infection and enable safe and prompt cessation of antibiotics. Such diagnostics are particularly needed for pneumonia, which is responsible for a substantial proportion of antibiotic misuse and is a well-established driver of resistance (6–8).

In this issue of the *Journal*, Walter and colleagues (pp. 1225–1237) take an important step toward solving this problem in the ICU (9). To do so, they make use of the defining host immune response in bacterial pneumonia, namely, neutrophilic alveolitis. Indeed, most of the clinical manifestations of pneumonia stem from this process, including 1) respiratory symptoms such as cough and purulent sputum; 2) systemic signs such as fever, which results from inflammatory signals derived in part from polymorphonuclear cells; and 3) radiographic infiltrates, which in pneumonia represent pus in the lung. Although relatively nonspecific, the BAL

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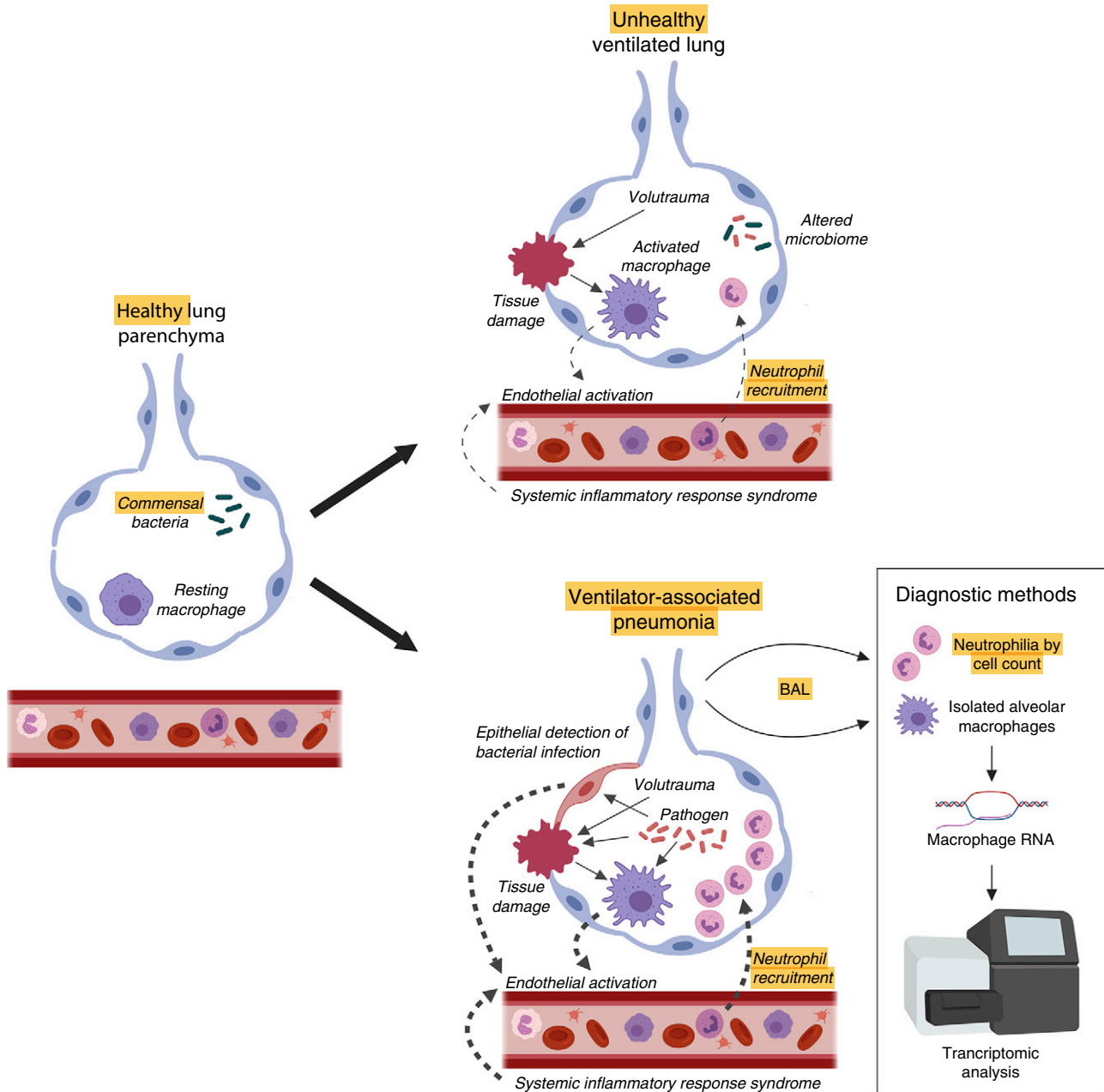


Figure 1. Pneumonic immune responses. Under healthy conditions (left panel), low levels of commensal bacteria and resting, noninflammatory macrophages predominate. The mechanically ventilated lung (top right panel) has numerous immune stimuli, for example, relating to volutrauma-induced tissue damage and systemic inflammation. These factors lead to endothelial activation and immune infiltrate, but not overt pneumonia. During ventilator-associated pneumonia (depicted in the bottom right panel), pathogenic bacteria further activate macrophages and induce both damage and stimulation of epithelial cells. Together, these drive a robust pyogenic inflammatory response, which may be detected by either one of the diagnostic methods described here.

neutrophilia test is believed to be highly sensitive for pneumonia, as previously shown in critically ill (10) and nonneutropenic immunocompromised patients (11).

In keeping with this canonical pathophysiologic principle, Walter and colleagues demonstrate here that nonbronchoscopic BAL (NBBAL) neutrophil counts of <50% provided a negative predictive value of 91.5% in patients with suspected ventilator-associated pneumonia, and coupled with a negative gram stain,

the negative predictive value approached 100%. Diagnosis of pneumonia was confirmed by quantitative culture, a method that is highly stringent but not widely available.

This is a remarkable finding in terms of not only test performance but also practicality. NBBAL does not require pulmonologists; it may be performed by trained respiratory therapists, which reduces the cost and potential delay involved with formal bronchoscopy, with no decrease in diagnostic yield (12).

Furthermore, the laboratory analytics required—cell counts and Gram stain—are universally available, inexpensive, and rapid. For these reasons, the current findings have the potential to make a broad and immediate impact on clinical practice in the ICU.

The diagnostic insufficiency of BAL neutrophilia as a standalone test is worth discussing. One explanation may relate to a phenomenon that is observed in advanced sepsis and is known as neutrophil paralysis, wherein polymorphonuclear cell extravasation and chemotaxis are impaired (13). Thus, the 8.5% of patients in the study who had bacterial infection without neutrophilia may have had insufficient recruitment of neutrophils into the alveolar space due to sepsis-induced immunosuppression. Fortunately, these few cases could be identified via Gram stain.

Walter and colleagues also present a provocative series of genomic studies in which they assessed the ability of alveolar macrophage transcriptomes to predict the diagnosis of ventilator-associated pneumonia. Because alveolar macrophages serve as the central innate immune sensor of alveolar bacteria and principal coordinator of neutrophil recruitment, one might predict their transcriptional status to be a highly accurate reporter of lung infection. Surprisingly, however, the operating characteristics of an RNA sequencing–based test did not significantly outperform the NBBAL neutrophilia test alone. A number of reasons may explain this finding.

First, alveolar macrophages do not operate in isolation in the generation of pneumonic immune responses (Figure 1). Numerous other components of the innate immune system contribute to pathogen detection in the lung, including epithelial cells, innate lymphocytes, platelets, and plasma constituents (e.g., complement) (14). Indeed, immune signaling in epithelial cells alone was shown to be sufficient to recruit neutrophils and control infection in a murine model of *Pseudomonas* pneumonia (15). Therefore, alveolar macrophage–independent neutrophilic responses may help to explain the findings reported here.

Second, alveolar macrophages process immune signals from not only microbes (Figure 1) but also systemic inflammatory sources and damaged tissue (induced by pathogens, ventilator-induced volutrauma, etc.). It may be that the integration of these various stimuli produces genomic responses that are simply more similar than they are different. Studies in pneumonia and sepsis support this hypothesis and further indicate that the ability to distinguish features such as the site of infection, microbial etiology, and even the presence of infection appears to diminish as the illness progresses (16).

Thus, in patients with advanced critical illness, such as those studied here, host transcriptomics may be more challenging to interpret. Perhaps sampling patients earlier, combining transcriptomics with other biomarkers (including the alveolar neutrophilia described here), or synthesizing host genomics with microbiomic sequencing would lead to further diagnostic refinement. Further omics-based investigations of such questions are certainly warranted.

Although it is surprising that rapid, inexpensive, and widely available tests such as BAL cell count and Gram stain perform as well as cutting-edge personalized techniques like RNA sequencing, the finding is all the more valuable given the practical advantages of the former. Prospective trials are needed to demonstrate that the algorithm presented here can indeed reduce antibiotic use without compromising patient safety, but these results should certainly encourage more studies on the

implementation of NBBAL sampling in the ICU. Finally, we submit that the present work, which describes a cost-effective rule-out test for infection inspired by sound pathophysiology, represents an important model for future studies aimed at reducing the unnecessary use of antibiotics—an often underappreciated but nonetheless rampant problem with potentially dire consequences for both society and the patient. ■

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Samir Gautam, M.D., Ph.D.
Lokesh Sharma, Ph.D.
Charles S. Dela Cruz, M.D., Ph.D.
Section of Pulmonary, Critical Care, and Sleep Medicine
Yale University School of Medicine
New Haven, Connecticut

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Developmental Milestones in Pediatric Research: A Case for Including Efficacy as Part of Interventional Trials in Infants with Cystic Fibrosis

In a study reported in this issue of the *Journal*, Stahl and colleagues (pp. 1238–1248) provide new evidence for the safety, tolerability, and potential efficacy of inhaled 6% hypertonic saline in infants with cystic fibrosis (CF) (1). PRESIS (Preventive Inhalation of Hypertonic Saline in Infants with Cystic Fibrosis), a randomized, double-blinded trial, included just over 40 subjects enrolled at an average age of 3 months and followed for 1 year after being assigned to inhalation of twice daily nebulized isotonic versus hypertonic saline. No subject experienced a study-related serious adverse event or withdrew from the trial for intolerance. Studies done in children so young are often limited to safety and tolerability, but the PRESIS investigators were able to move beyond this and include measures of potential benefit to pulmonary health.

The study found that 6% hypertonic saline nebulized twice a day for 52 weeks provided a statistically significant improvement in lung function as measured by the primary efficacy outcome, lung clearance index (LCI). When compared with infants assigned to isotonic saline, those given 6% saline experienced a reduction (i.e., improvement) in LCI that was sustained over the 12-month observational period. The improvement in LCI, although not large, is similar to the difference reported between healthy children and those with CF at this age (2, 3). Larger improvements in LCI were seen in a small number of preschool-aged children after they started ivacaftor, but most of the children had higher (i.e., worse) baseline LCI values, and the few patients with normal baseline LCI tended to show little change with therapy (4).

The feasibility and successful use of LCI in the PRESIS study is perhaps as informative as the results. This is the first publication to report the successful use of LCI in a multicenter, randomized controlled clinical trial in infants. A single-center pilot substudy within the Infant Study of Inhaled Saline trial previously demonstrated that subjects receiving inhaled 7% saline had better LCI values than those receiving isotonic saline, but the participants had a median age of >2 years at enrollment (5). Stahl and colleagues now extend the argument that LCI can identify the health impacts of pulmonary interventions in trials that include very young children

across multiple centers. Although the clinically meaningful change in LCI at this age is uncertain, they should be commended for this work requiring repeated study-related procedures for both LCI and chest magnetic resonance imaging (MRI).

There are a number of interesting observations beyond the key findings reported in this study. The PRESIS trial was set up to take advantage of early diagnosis of CF after newborn screening, with the goal of delaying or reducing the development of CF lung disease. This demonstrates the need for sensitive measures such as LCI to detect relatively small differences in lung function when focused on very young patients who may have been diagnosed before the onset of any pulmonary symptoms. Such measures were not available in initial studies of the effect of newborn screening on CF lung disease (6). Improvement in LCI was not associated with improvement in lung morphology as measured by MRI or the risk of predefined acute pulmonary exacerbations. Caution against overinterpreting data from a study of this size is necessary, but the findings suggest that either lung functional and structural abnormalities at this age do not align or lung imaging outcome measures may have less value in a population this young. Clearly, alternative interventions or imaging modalities may produce different results, but prior research also found a poor correlation between results from LCI and computed tomography imaging in subjects under 1 year old (7, 8). Less than one-third of children with CF appear to have bronchiectasis on computed tomography at 1 year of age (9).

The modified Fuchs criteria were used to define acute pulmonary exacerbation in this trial. This definition is a common, valuable tool in CF clinical research (10), but it may be less useful in such young children. One recognizes that infants, even when acutely ill, often lack many of the symptoms required by the Fuchs criteria (e.g., change in sputum, hemoptysis, dyspnea, sinus pain, chest X-ray changes, and change in lung function), which may explain why less than half of the subjects in the trial experienced a protocol-defined exacerbation over the 12-month period. Although hypertonic saline may not significantly impact the risk of acute pulmonary exacerbations in young children (11), this also underscores the ongoing need to consider alternative quantitative efficacy measures for the youngest populations. The CF community has recently been encouraged, if not surprised, by potentially important benefits reported in studies testing CFTR (cystic fibrosis transmembrane conductance regulator) modulator drugs in infants and toddlers with CF (12). Thus, accumulating evidence suggests that using chronic preventive therapies before the onset of observable symptoms may provide clinically meaningful benefits. More work is needed to understand long-term safety and whether

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Multidimensional Assessment of the Host Response in Mechanically Ventilated Patients with Suspected Pneumonia

James M. Walter^{1*}, Ziyou Ren^{1*}, Tyrone Yacoub², Paul A. Reyfman¹, Raj D. Shah¹, Hiam Abdala-Valencia¹, Kiwon Nam¹, Vince K. Morgan¹, Kishore R. Anekalla¹, Nikita Joshi¹, Alexandra C. McQuattie-Pimentel¹, Ching-I Chen¹, Monica Chi¹, SeungHye Han¹, Francisco J. Gonzalez-Gonzalez¹, Saul Soberanes¹, Raul P. Aillon¹, Satoshi Watanabe¹, Kinola J. N. Williams¹, Ziyun Lu¹, Joseph Paonessa¹, Peter Hountras¹, Madonna Breganio¹, Nicole Borkowski¹, Helen K. Donnelly¹, Jonathan P. Allen³, Luis A. Amaral², Ankit Bharat⁴, Alexander V. Misharin¹, Neda Bagheri², Alan R. Hauser³, G. R. Scott Budinger^{1*}, and Richard G. Wunderink^{1*}

¹Division of Pulmonary and Critical Care Medicine, Department of Medicine, and ⁴Division of Thoracic Surgery, Department of Surgery, Feinberg School of Medicine, and ³Department of Microbiology and Immunology, Northwestern University, Chicago, Illinois; and ²Department of Chemical and Biological Engineering, Northwestern University, Evanston, Illinois

ORCID IDs: 0000-0001-7428-3101 (J.M.W.); 0000-0002-6435-6001 (P.A.R.).

Abstract

Rationale: The identification of informative elements of the host response to infection may improve the diagnosis and management of bacterial pneumonia.

Objectives: To determine whether the absence of alveolar neutrophilia can exclude bacterial pneumonia in critically ill patients with suspected infection and to test whether signatures of bacterial pneumonia can be identified in the alveolar macrophage transcriptome.

Methods: We determined the test characteristics of alveolar neutrophilia for the diagnosis of bacterial pneumonia in three cohorts of mechanically ventilated patients. In one cohort, we also isolated macrophages from alveolar lavage fluid and used the transcriptome to identify signatures of bacterial pneumonia. Finally, we developed a humanized mouse model of *Pseudomonas aeruginosa* pneumonia to determine if pathogen-specific signatures can be identified in human alveolar macrophages.

Measurements and Main Results: An alveolar neutrophil percentage less than 50% had a negative predictive value of greater than 90% for bacterial pneumonia in both the retrospective ($n = 851$) and validation cohorts ($n = 76$ and $n = 79$). A transcriptional signature of bacterial pneumonia was present in both resident and recruited macrophages. Gene signatures from both cell types identified patients with bacterial pneumonia with test characteristics similar to alveolar neutrophilia.

Conclusions: The absence of alveolar neutrophilia has a high negative predictive value for bacterial pneumonia in critically ill patients with suspected infection. Macrophages can be isolated from alveolar lavage fluid obtained during routine care and used for RNA-Seq analysis. This novel approach may facilitate a longitudinal and multidimensional assessment of the host response to bacterial pneumonia.

Keywords: bacterial pneumonia; host response; alveolar macrophages; RNA-Seq

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*These authors contributed equally.

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At a Glance Commentary

Scientific Knowledge on the

Subject: Analysis of lower respiratory tract specimens in patients with suspected pneumonia has largely focused on pathogen detection. Assessment of the host response is recognized as an important yet understudied approach to aid pneumonia diagnosis and management.

What This Study Adds to the

Field: Assessment of alveolar neutrophilia can help to rapidly exclude bacterial pneumonia in critically ill patients with a negative predictive value approaching 100% when an alveolar neutrophil percentage of less than 50% is paired with a negative lavage fluid gram stain. In addition, we demonstrate the feasibility of measuring changes in host gene expression to refine pneumonia diagnosis using alveolar macrophages from BAL samples obtained during routine clinical care. This novel approach may facilitate a longitudinal and multidimensional assessment of the host response to bacterial pneumonia.

Antibiotic overuse is an urgent public health problem (1, 2). The frequent use of broad-spectrum antibiotic therapy puts patients at risk for infection with multidrug-resistant pathogens and increases antimicrobial resistance within the healthcare system (3, 4).

Pneumonia is a leading cause of death and the most common infection identified in patients admitted to the ICU (5–7). Diagnostic uncertainty regarding both the presence and etiology of pneumonia contributes to the frequent administration of antibiotic therapy to uninfected patients. Clinical variables such as fever, sputum production, and leukocytosis are insufficient to reliably guide management decisions (8). Improved diagnostic approaches are urgently needed to help minimize antibiotic exposure for uninfected patients.

Rapid diagnostic testing in patients with suspected pneumonia has focused primarily on pathogen detection (9). However, there is growing recognition that pneumonia pathogenesis is driven as much by host-specific factors as by the invading pathogen (10). Diagnostic tests based on the host response to infection may therefore improve pneumonia diagnosis and management.

Bacterial pneumonia is characterized by the rapid influx of neutrophils into the alveolar space in immunocompetent patients (11). The alveolar space can be safely sampled during routine clinical care using nonbronchoscopic bronchoalveolar lavage (NBBAL) (12). Assessment of alveolar neutrophilia provides a rapid and inexpensive means of assessing a key immune response to bacterial pneumonia. Although a low neutrophil percentage on cell count analysis has been reported to have a high negative predictive value for bacterial pneumonia, these data are based on a limited number of small studies (8, 13–15).

Next-generation sequencing technologies, including RNA-Seq, allow for a detailed analysis of the host response to infection. As RNA-Seq and PCR techniques are based on similar chemistry, predictive

gene signatures identified in an unbiased fashion with RNA-Seq could be rapidly incorporated into PCR-based diagnostic tests currently being used for pathogen detection.

In this study, we evaluate whether the absence of alveolar neutrophilia can exclude bacterial pneumonia in retrospective and prospective cohorts of critically ill patients with suspected infection. We then pair flow-cytometry sorting of lavage fluid with RNA-Seq to investigate whether the transcriptome of a key lung immune cell, the alveolar macrophage, can be used to discriminate patients with and without bacterial pneumonia. Finally, we use a humanized mouse model of *Pseudomonas aeruginosa* pneumonia to determine if pathogen-specific signatures can be identified in human alveolar macrophages. Our results show that the absence of alveolar neutrophilia, especially if paired with a negative gram stain, can help to rapidly exclude the diagnosis of bacterial pneumonia with encouraging test characteristics. In addition, we demonstrate how RNA-Seq can be used to identify transcriptome-level changes in alveolar macrophages using samples obtained during routine clinical care. This approach may dramatically improve our ability to study dynamic changes in host gene expression over the course of infection. Some of the results of these studies have been previously reported in the form of abstracts (16, 17).

Methods

Patients

In a retrospective analysis, we analyzed BAL specimens obtained as part of routine care

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Correspondence and requests for reprints should be addressed to James M. Walter, M.D., Northwestern University, Feinberg School of Medicine Division of Pulmonary and Critical Care Medicine, 240 East Huron Street, Room M-300, Chicago, IL 60611. E-mail: james.walter@northwestern.edu.

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from patients undergoing evaluation for ventilator-associated pneumonia at an urban academic medical center between January 1, 2010 and June 30, 2013. Bronchoscopic BAL specimens were collected by intensivists. NBBAL specimens were collected by respiratory therapists using a 16-F sampling catheter (BALCath; Kimberly-Clark Corporation). A volume of at least 60 ml of nonbacteriostatic saline was instilled into the right or left lung as determined by the treating physician. The initial NBBAL aliquot was discarded as part of routine practice to minimize the contribution of central airway colonization. For bronchoscopic BAL, an initial aliquot is not routinely discarded by all operators. Cell counts were performed through standard automated Coulter counter technology. Cell differentials were determined manually by a clinical laboratory technologist. BAL neutrophils were recorded as a percentage of the cells in the lavage fluid.

Subsequently, in two independent prospective cohorts, we enrolled mechanically ventilated adult patients aged 18 years or older in the ICU in whom a bronchoscopic BAL or NBBAL was performed to investigate suspected pneumonia from December 1, 2015 to

January 4, 2017. In one of these cohorts, a small aliquot (1–5 ml) of lavage fluid was obtained for flow-cytometry sorting as part of study protocols approved by the Northwestern University Institutional Review Board. Fluid was stored in 15-ml conical tubes (Corning) at 4°C until processing.

In all cohorts, BALs with greater than or equal to 12% bronchial epithelial cells were excluded. Patients who were neutropenic (defined as a serum absolute neutrophil count < 1,000 cells/ μ l) or who had bronchiectasis or cystic fibrosis were also excluded. In the prospective cohorts, patients could be enrolled more than once if each lavage was collected for a distinct episode of suspected pneumonia during the index hospitalization or if they were rehospitalized with concern for a new infection.

Pneumonia Definition

The definition of bacterial pneumonia was based on recent consensus guidelines (18, 19). Specifically, pneumonia was defined as greater than or equal to 10^4 cfu/ml of a bacterial species on quantitative culture in the setting of a clinical suspicion of infection (identified by the treating clinician ordering a bronchoscopic BAL or

NBBAL with bacterial culture) and an abnormal chest radiograph. For patients with a recent (<48 h) change in antibiotics at the time of sampling, bacterial growth of greater than or equal to 10^3 cfu/ml was considered positive, similar to previous trials (20). Calibrated loop technology was used for quantitative cultures.

Lavage Fluid Processing and Cell Sorting

All lavage samples were processed for flow-cytometry sorting within 12 hours of collection using a modification of a previously described procedure, as detailed in the online supplement (21, 22). The gating strategy used is shown in Figure 1.

Humanized Mouse Pneumonia Model

The Institutional Animal Care and Use Committee and Institutional Review Board (IS00000747) at Northwestern University approved all animal procedures. MISTRG mice (Jax 017712) were reconstituted with CD34⁺ stem cells isolated from human cord blood obtained from a commercial vendor (StemCell Technologies) (23). Fourteen-week-old mice with confirmed reconstitution were intratracheally inoculated with either a low-virulence (PABL065) or

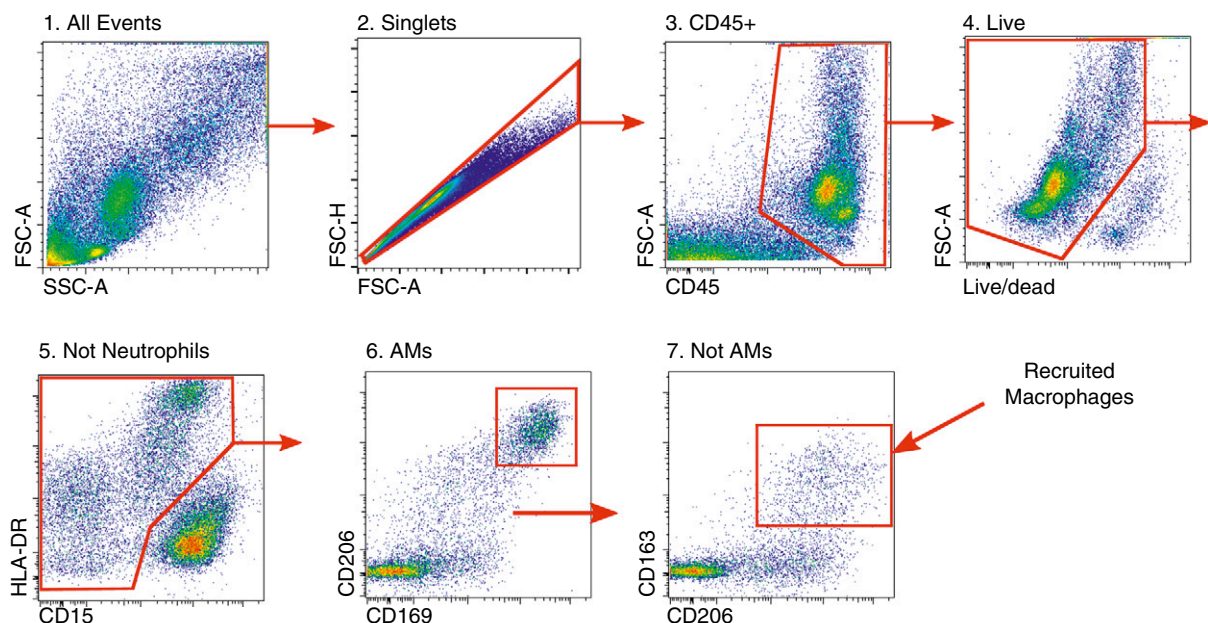


Figure 1. Gating strategy used to identify resident and recruited macrophages from patient BAL fluid. Tissue-resident alveolar macrophages were identified using sequential gating as singlets/CD45⁺/live/CD15⁻/CD206⁺/CD169⁺/HLA⁻DR⁺/high autofluorescence; recruited macrophages were identified as singlets/CD45⁺/live/CD15⁻/CD169⁻/CD206⁺/CD163⁺/HLA-DR⁺/high autofluorescence. Data from a representative patient are shown. AMs = alveolar macrophages; CD = cluster of differentiation; FSC-A = forward scatter-area; FSC-H = forward scatter-height; SSC-A = side scatter-area.

high-virulence (PABL012) clinical strain of *P. aeruginosa*. Four hours after infection, lungs were harvested for flow sorting of alveolar macrophages. Full details of the experimental procedure are provided in the online supplement.

RNA Isolation and Sequencing

RNA isolation and sequencing procedures are detailed in the online supplement.

Bioinformatics Analysis

Bioinformatic analyses are detailed in the online supplement.

Statistics

Data are expressed as mean and SD or median and interquartile range (IQR) according to data distribution. BAL neutrophil and procalcitonin distributions were compared between patients with and without bacterial pneumonia using the Mann-Whitney *U* test. Receiver operating characteristic (ROC) curves were constructed for alveolar neutrophilia and procalcitonin to discriminate between patients with and without bacterial pneumonia. The area under the curve (AUC), sensitivity, specificity, positive predictive value, negative predictive value (NPV), positive likelihood ratio, and negative likelihood ratio were calculated.

For predictive modeling, least absolute shrinkage and selection operator (LASSO) was fit to the top 50 differentially expressed genes ordered by false discovery rate (FDR) *q* value in the resident and recruited macrophage datasets. A summary of statistics with LASSO was calculated using the R package GLMNET (24). Tenfold cross-validation was performed to determine the most suitable parameters for the prediction model for each cell type. The average mean squared error and 95% confidence interval (CI) were reported from the most accurate model derived from 11 cross-validation runs. An ROC curve was generated for each cell type using the R package pROC and the AUC was calculated. Weighted correlation network analysis was performed using log counts per million and clinical variables (25, 26).

Analysis was performed using GraphPad Prism (version 7.02, GraphPad Software) and R (version 3.4.1).

Results

Predictive Value of BAL Neutrophilia

Clinical characteristics of patients are shown in Table 1. Bacterial pneumonia was diagnosed in more than one-third of each cohort. The majority of patients were

receiving antibiotics at the time of BAL (81% in the retrospective cohort, 86% in prospective cohort A, and 100% in prospective cohort B). In-hospital mortality was 26% in the retrospective cohort, 37% in prospective cohort A, and 37% in prospective cohort B.

In the retrospective cohort, 1,156 BALs were screened for inclusion. After exclusion of 305 samples (53 with $\geq 12\%$ bronchial epithelial cells, 126 due to repeat sampling, 104 from patients with a serum absolute neutrophil count $< 1,000$ cells/ μL , and 22 for normal chest imaging), 851 were included in the final analysis. Of these, 344 (40.4%) met the definition for bacterial pneumonia. The most common pathogens isolated were: *P. aeruginosa* (90, 26.2%), methicillin-resistant *Staphylococcus aureus* (30, 8.7%), and *Klebsiella pneumoniae* (30, 8.7%). A full list of isolated pathogens from all cohorts is included in the online supplement. Receiver operating characteristics for BAL neutrophilia as a predictor of bacterial pneumonia are shown in Figure 2. The AUC for BAL neutrophilia was 0.751 (95% CI, 0.719–0.784). When BAL neutrophils were less than 50%, the NPV for pneumonia was 91.5%. The operating characteristics for various thresholds of BAL neutrophil percentage are shown in Table 2.

As antibiotic administration may impact the microbiologic yield of BAL, we then restricted our analysis to the 179 patients who were not receiving antibiotics at the time of sampling. In this cohort, the AUC for alveolar neutrophilia for the diagnosis of bacterial pneumonia was 0.767 (95% CI, 0.697–0.837). A BAL neutrophil cutoff of less than 50% had a sensitivity of 86.7%, specificity of 56.5%, positive predictive value of 56.5%, and NPV of 86.7%.

In our prospective cohorts, 76 samples were included in cohort A and 79 samples in cohort B. Twenty-eight samples (36.8%) met the definition for bacterial pneumonia in cohort A and 29 (36.7%) in cohort B. A BAL neutrophil threshold of less than 50% had an NPV of 90.5% in cohort A and 90.9% in cohort B (see Table E3 in the online supplement for full operating characteristics). When the analysis was restricted to patients who were either not receiving antibiotics, receiving antibiotics that did not cover the pathogen that was ultimately isolated, or did not have a change in antibiotics within 48 hours of sampling, a BAL neutrophil threshold of

Table 1. Characteristics of Study Patients

	Retrospective Cohort (851 Patients)	Prospective Cohort A (73 Patients, 76 Unique Episodes of Suspected Pneumonia)	Prospective Cohort B (68 Patients, 79 Unique Episodes of Suspected Pneumonia)
Male	500 (58)	42 (55)	39 (57)
Age, yr	61.4 (50–70)	61 (54–71)	63.5 (53–73)
White	467 (55)	33 (76)	32 (40)
Pneumonia type			
CAP	0 (0)	22 (29)	20 (25)
HAP/VAP	851 (100)	54 (71)	59 (75)
APS score on ICU admission	67 (46–91)	53 (33–76)	61 (48–85)
On antibiotics before BAL	692 (81)	65 (86)	79 (100)
Bacterial pneumonia	344 (40)	28 (37)	29 (37)
ICU length of stay, d	15.6 (7–28)	11 (6–20)	13.5 (7–26)
In-hospital mortality	225 (26)	28 (37)	25 (37)

Definition of abbreviations: APS = Acute Physiology Score; CAP = community-acquired pneumonia; HAP = hospital-acquired pneumonia; VAP = ventilator-associated pneumonia. Data are presented as *n* (%) or median (interquartile range).

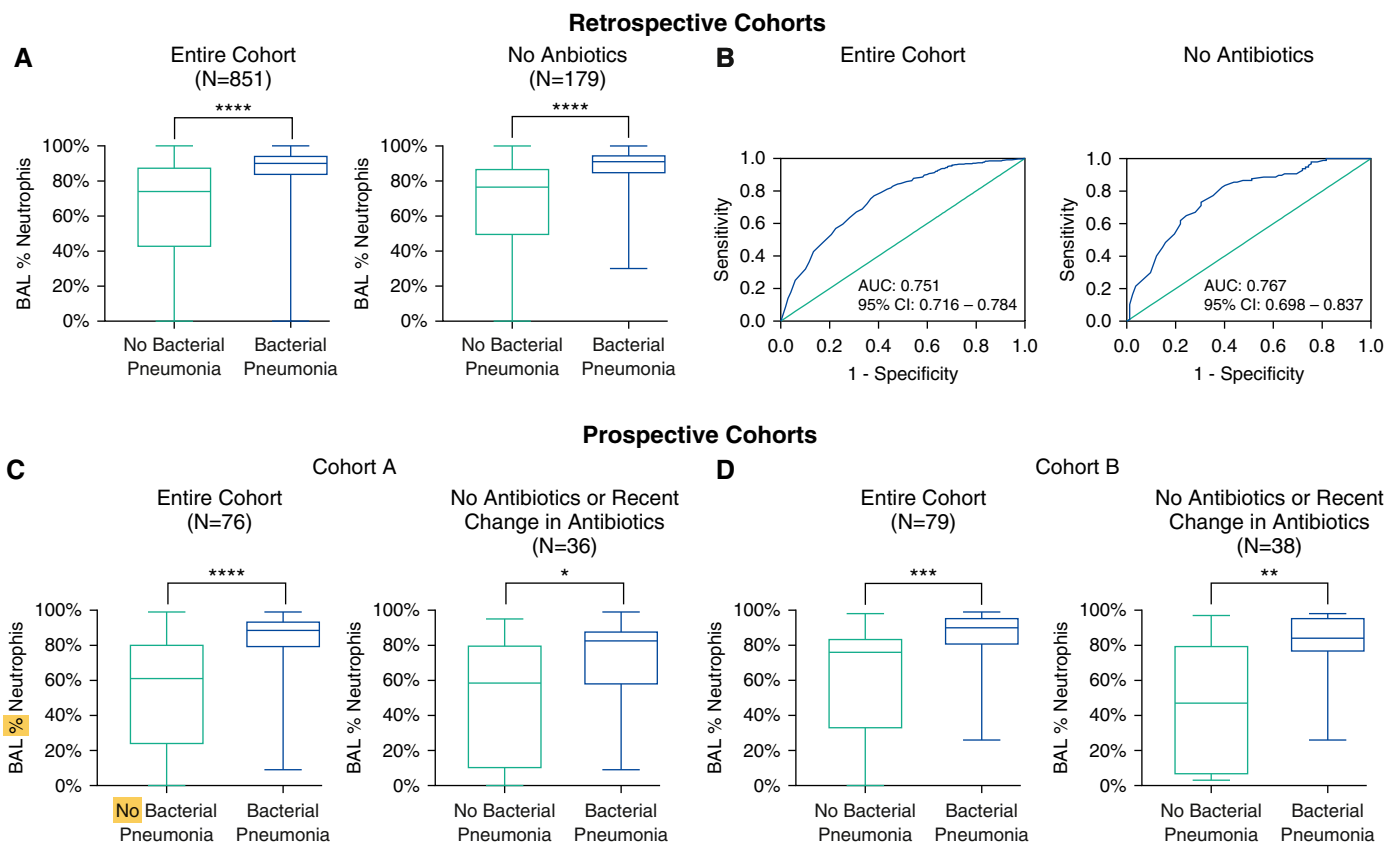


Figure 2. BAL neutrophil percentage has a high negative predictive value for bacterial pneumonia in critically ill patients with suspected infection. (A) Percent of neutrophils in BAL fluid in a retrospective cohort of mechanically ventilated patients with suspected pneumonia stratified by antibiotic use at the time of sampling. Boxes extend from the 25th to the 75th percentiles of recorded values, and the horizontal line is plotted at the median. Vertical lines represent ranges. (B) Receiver operating characteristics curve of BAL neutrophil percentage for the diagnosis of bacterial pneumonia stratified by antibiotic use at the time of sampling. (C and D) Percentage of neutrophils in BAL fluid in two independent prospective cohorts of mechanically ventilated patients with suspected pneumonia. Results are shown for the entire cohort and the subgroup of patients who were either not on antibiotics or did not have a change in antibiotics within 48 hours of sampling. Patients who were receiving antibiotics that did not cover the isolated pathogen were considered to be off antibiotics. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. AUC = area under the curve; CI = confidence interval.

less than 50% retained a high NPV: 85.7% in cohort A (40 samples) and 86.7% in cohort B (38 samples). We repeated our analysis of our prospective cohorts using a bronchial epithelial cell cutoff of greater than 5% to identify contaminated samples. This did not significantly change our results (data not shown).

We then compared the test characteristics of BAL neutrophilia to procalcitonin—a commonly used biomarker to discriminate bacterial from nonbacterial lower respiratory tract infections (Figure 3) (27, 28). This analysis included 61 patients from prospective cohort A (80% of the cohort) and 60 patients from prospective cohort B (76% of the cohort) who had a procalcitonin obtained within 6 hours of alveolar sampling. In both cohorts, procalcitonin was a poor predictor of bacterial

pneumonia. There was no significant difference in procalcitonin values between patients with and without bacterial pneumonia in either cohort. The AUC for bacterial pneumonia was 0.550 (95% CI, 0.393–0.707) in cohort A and 0.566 (95% CI, 0.413–0.720) in cohort B.

Finally, we asked whether pairing a BAL neutrophil threshold of less than 50% with a negative BAL gram stain (another inexpensive and rapidly available test) could further improve the NPV for bacterial pneumonia. This combination had an NPV for bacterial pneumonia of 100% in prospective cohort A and 95.0% in prospective cohort B.

RNA-Seq Analysis of Flow-sorted Macrophages

As no BAL neutrophil cutoff could exclude bacterial pneumonia with certainty, we

explored whether gene expression profiling of alveolar macrophages might further discriminate between patients with and without pneumonia. Accordingly, we performed RNA-Seq analysis of flow-sorted resident and recruited macrophages obtained from patients in prospective cohort B.

Nine samples were excluded from our analysis on the basis of our described filtering criteria. Estimation of differential gene expression was performed in a derivation cohort, which included 42 flow-sorted resident alveolar macrophage samples (15 from patients with pneumonia and 27 from patients without pneumonia) and 44 recruited macrophage samples (14 from patients with pneumonia and 30 from patients without pneumonia). Using an FDR q value less than 0.05, 33 differentially expressed genes were identified in resident

Table 2. Operating Characteristics of BAL Neutrophil Percentage to Identify the Presence of Bacterial Pneumonia in Mechanically Ventilated Patients with Suspected Infection

BAL Neutrophils (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Positive LR (95% CI)	Negative LR (95% CI)
≥40	96.8	23.9	46.2	91.6	1.27 (1.2–1.3)	0.14 (0.1–0.3)
≥50	95.9	29.8	48.1	91.5	1.37 (1.3–1.5)	0.14 (0.1–0.2)
≥60	92.7	35.7	49.4	87.8	1.44 (1.3–1.6)	0.20 (0.1–0.3)
≥70	88.1	44.4	51.8	84.6	1.58 (1.5–1.7)	0.27 (0.2–0.4)
≥80	79.1	59.0	56.7	80.6	1.93 (1.7–2.2)	0.35 (0.3–0.4)
≥90	48.5	82.6	65.4	70.3	2.79 (2.2–3.5)	0.62 (0.6–0.7)

Definition of abbreviations: CI = confidence interval; LR = likelihood ratio; NPV = negative predictive value; PPV = positive predictive value. Retrospective cohort ($n = 851$).

alveolar macrophages and 24 differentially expressed genes were identified in recruited macrophages (Figures 4A and 4B). Functional enrichment analysis with GO Biological Processes revealed common infection-related processes including Defense Response, Immune System Process, and Response to Molecule of Bacterial Origin in both sorted-cell populations (Figures 4C and 4D).

Although functional enrichment analysis is helpful to identify broad pathophysiologic categories within a given gene set, the processes identified are not specific to lower respiratory tract infections. We therefore performed gene set enrichment analysis using the curated Comparative Toxicogenomics Database pneumonia gene set to assess whether our transcriptional data were enriched for genes

known to be associated with pneumonia (29, 30). Significant enrichment for the curated gene set was found among differentially expressed genes between patients with and without pneumonia in both cell types (Figures 4E and 4F). Two previously published gene signatures associated with bacterial pneumonia, both generated from analysis of peripheral blood, were not identified in our dataset (31, 32).

We then used LASSO regression to generate a predictive model of bacterial pneumonia for both resident and recruited macrophages using our training dataset. The predictive gene signatures identified were then applied to a validation cohort, which included 20 resident macrophage samples (9 from patients with pneumonia and 11 from patients without pneumonia) and 11 recruited macrophage samples (7 from patients with pneumonia and 4 from patients without pneumonia). The AUC for the three-gene signature (TNFAIP3, WSB1, and PFKFB3) identified in resident alveolar macrophages was 0.78 (95% CI, 0.56–0.99). The AUC for the five-gene signature (MMP14, TNFAIP3, NFKBIZ, TNFAIP6, and HSP90AA1) identified in recruited macrophage samples was 0.74 (95% CI, 0.56–1.00).

As we were underpowered to focus on any one specific pathogen, we performed correlation analysis using weighted correlation network analysis to determine whether clinical variables including subtypes of isolated pathogens might be associated with gene expression in our dataset (Figure 5). In both resident and recruited macrophages, the cluster of genes most strongly associated with bacterial pneumonia was also highly associated with BAL neutrophilia. In resident alveolar macrophages, this cluster included 319 genes strongly associated with both BAL

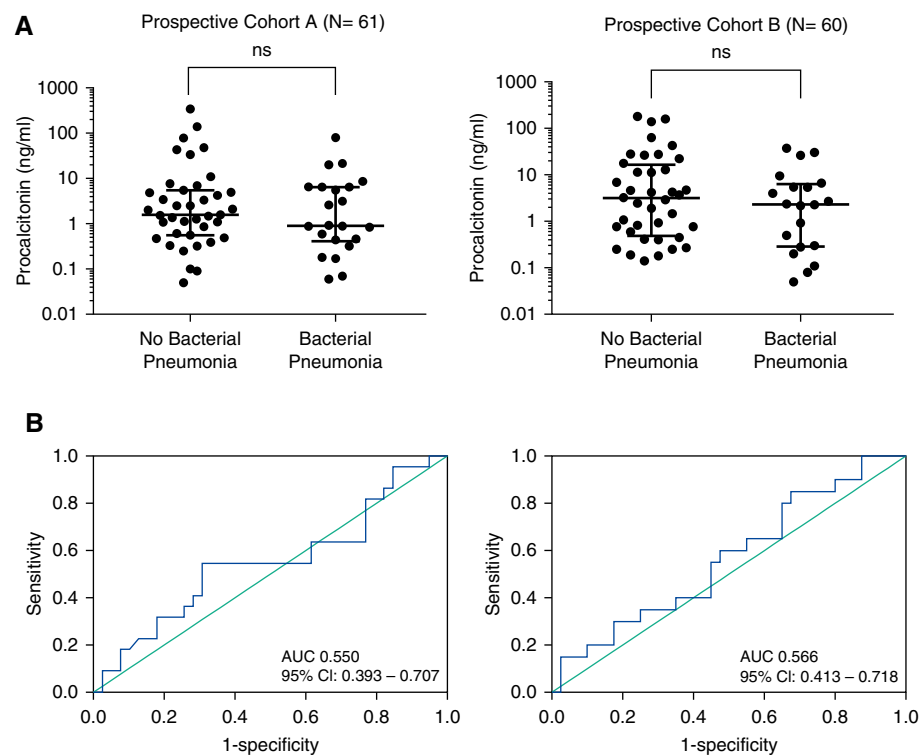


Figure 3. Procalcitonin does not discriminate between the presence or absence of bacterial pneumonia in mechanically ventilated patients with suspected infection. (A) Scatter plots showing procalcitonin values in two independent prospective cohorts of mechanically ventilated patients with suspected pneumonia. Vertical lines extend from the 25th to the 75th percentiles of recorded values, and the horizontal line is plotted at the median ($P > 0.05$ for both cohorts). (B) Receiver operating characteristics curves of procalcitonin for the diagnosis of bacterial pneumonia. AUC = area under the curve; CI = confidence interval; ns = not significant.

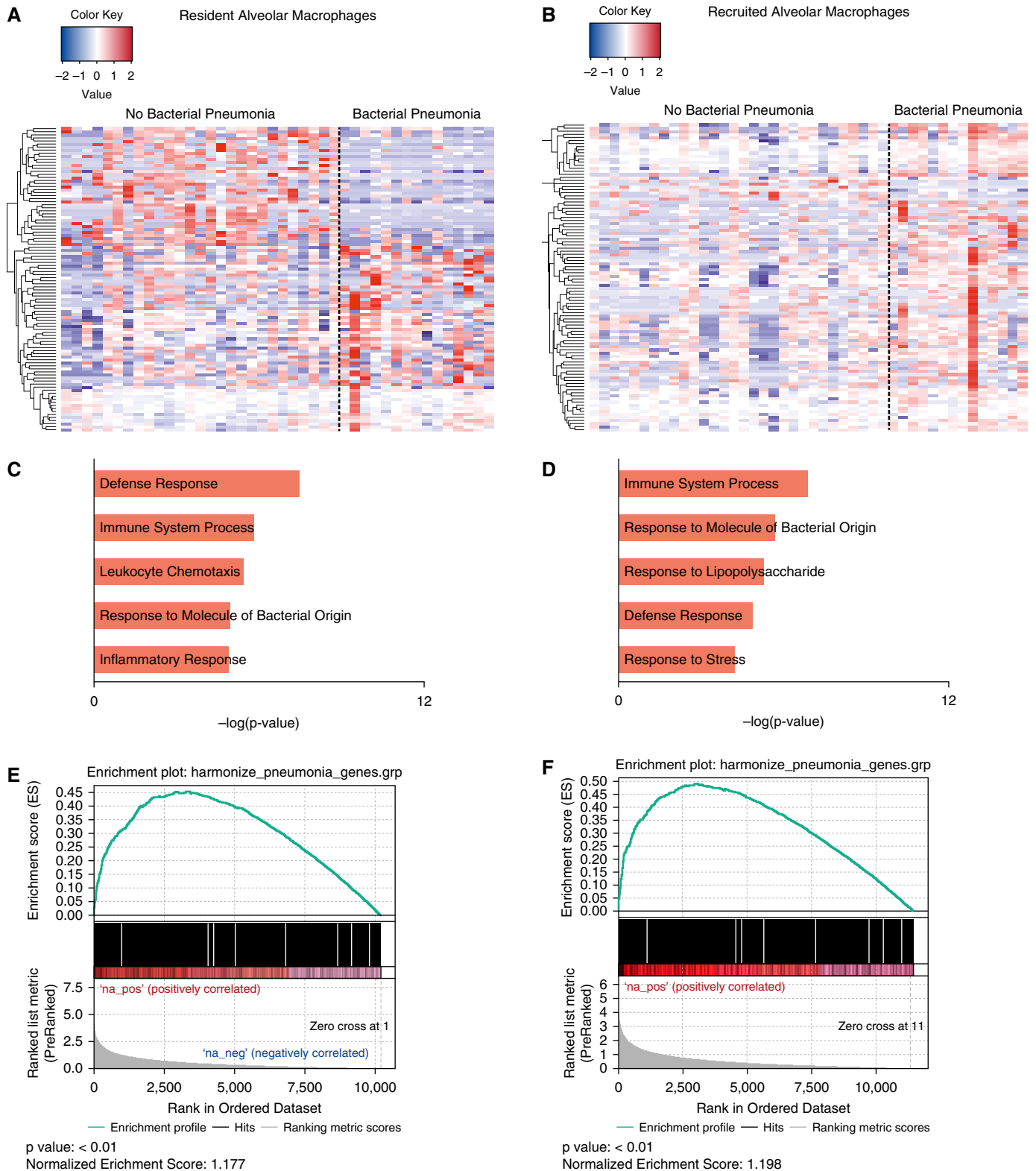


Figure 4. Transcriptional profiling of flow-sorted resident and recruited alveolar macrophages from critically ill patients with suspected infection reveals a signature of bacterial pneumonia. (A and B) In a derivation cohort, 49 flow-sorted resident alveolar macrophage samples and 46 recruited macrophage samples were obtained from critically ill patients with suspected infection and processed for RNA sequencing. Estimation of differential gene expression using EdgeR was performed comparing patients with and without pneumonia, and heatmaps were generated showing significantly differentially expressed genes ordered using hierarchical clustering. Columns represent individual patients, and rows represent specific genes. (C and D) Functional enrichment

neutrophilia ($P = 4 \times 10^{-7}$) and bacterial pneumonia ($P = 0.008$). Sixteen of these genes overlapped with those found in our differential expression analysis. Similarly, in recruited macrophages, a unique cluster of 220 genes was significantly associated with both BAL neutrophilia ($P = 5 \times 10^{-7}$) and bacterial pneumonia ($P = 0.002$). Twenty-five of these genes overlapped with genes identified in the differential expression analysis. A full list of overlapping genes is provided in the online supplement.

Humanized Mouse Model of *P. aeruginosa* Pneumonia

Macrophages exhibit remarkable plasticity, demonstrating distinct transcriptional responses to different pathogens (33). Identification of unique host response signatures might therefore facilitate the diagnosis of pneumonia caused by a specific pathogen. We compared the transcriptional response of human alveolar macrophages in a mouse lung environment (humanized mouse) during infection with two clinical strains of *P. aeruginosa*.

After intratracheal infection with either a low- or high-virulence strain of *P. aeruginosa*, human alveolar macrophages were isolated from murine whole-lung tissue and processed for RNA-Seq (Figures 6A–6D). Using *k*-means clustering ($k = 6$), several distinct gene clusters were identified, including a group of 711 genes upregulated in all infected mice, a cluster of 447 genes upregulated in the setting of infection with the low-virulence strain of *P. aeruginosa*, and a cluster of 279 genes unique to infection with the high-virulence strain. Overall, there were 1,925 differentially expressed genes (identified by an FDR q value < 0.05) between mice infected with the high-virulence strain of *P. aeruginosa* and control mice.

Of the 100 most differentially expressed genes in mice infected with the high-virulence strain, 89 were identified in alveolar macrophages isolated from a cohort of four mechanically ventilated patients with *P. aeruginosa* and four uninfected control subject. To approximate alveolar macrophage gene expression in the healthy human lung, we used alveolar macrophage RNA-Seq data from lung tissue obtained

from four age- and sex-matched donors at the time of lung transplantation as our controls (34). Humans with *P. aeruginosa* pneumonia demonstrated a similar gene expression pattern to infected MISTRG mice (Figure 6D).

Discussion

We demonstrate how an assessment of the host response can aid the diagnosis of bacterial pneumonia. First, we show in both retrospective and prospective cohorts that the absence of alveolar neutrophilia is useful in ruling out bacterial pneumonia in mechanically ventilated patients with suspected infection. Our study is the largest to determine the test characteristics of BAL neutrophilia in critically ill patients (8, 13–15). Second, we show how next-generation sequencing techniques can be applied to clinical samples to identify transcriptomic signatures of infection within a key lung immune cell, the alveolar macrophage. Finally, we provide proof of concept using a humanized mouse model of pneumonia that pathogen-specific transcriptional signatures can be identified. Our results support the identification of novel host response biomarkers to aid the diagnosis and treatment of pneumonia.

Current guidelines recommend empiric broad-spectrum antibacterial therapy for patients with suspected pneumonia and respiratory failure (18, 19). Because of this emphasis on early empiric therapy, patients in the ICU receive on average more than 1.5 doses of antibiotics per day (35). Antibiotic overuse promotes the emergence of resistant pathogens and leads to adverse patient outcomes (36, 37). Accordingly, biomarkers with high sensitivity are needed to identify patients in whom antibiotics can be safely avoided or rapidly discontinued. BAL neutrophilia is an appealing diagnostic test, as it is inexpensive, widely available, and reflective of a fundamental host immune response to bacterial pneumonia.

We show that the absence of BAL neutrophilia has a high NPV for bacterial pneumonia. A BAL neutrophil percentage of less than 50% had an NPV for bacterial pneumonia exceeding 90% in all cohorts.

When a BAL neutrophil threshold of less than 50% is paired with a negative BAL gram stain, the NPV for bacterial pneumonia approaches 100%. We believe the combination of these two rapid and inexpensive tests has the potential to facilitate the rapid discontinuation of empiric antibiotic therapy in many critically ill patients with suspected bacterial pneumonia. Importantly, we demonstrate that the test characteristics of BAL neutrophilia are minimally impacted by the administration of antibiotics. As the majority of critically ill patients with a suspected new pneumonia receive antibiotics before diagnostic testing, a biomarker that remains informative in this setting is particularly valuable (20, 38).

In both of our prospective cohorts, BAL neutrophilia outperformed procalcitonin, a peripheral blood biomarker used to help discriminate bacterial from nonbacterial infection (28). This observation is concordant with recent work demonstrating that disease-specific host response signatures are superior to systemic markers of inflammation (39). The results of our correlation analysis, in which the clusters of genes most strongly associated with bacterial pneumonia were also highly associated with BAL neutrophilia, further support the utility of this test.

As BAL neutrophilia is insufficient to exclude bacterial pneumonia with certainty, we asked whether a detailed assessment of the host response using next-generation sequencing might yield additional informative markers of bacterial pneumonia. Recent advances in flow cytometry allow for the unequivocal identification of myeloid cells in the human lung (21, 40, 41). When paired with BAL in patients with suspected pneumonia, these techniques enable individual immune cell populations to be isolated from the alveolus and processed for RNA-Seq.

We identified a transcriptional signature of infection in both resident and recruited macrophages from patients with bacterial pneumonia compared with those without infection. We were able to leverage this dataset to generate predictive gene signatures from both resident and recruited macrophages. These signatures identified

Figure 4. (Continued). analysis with GO Biological Processes was performed using GOrilla. Representative GO processes upregulated in patients with pneumonia are shown. (E and F) Gene set enrichment analysis was performed using the curated Comparative Toxicogenomics Database pneumonia gene set. Enrichment plots with *P* values and normalized enrichment scores for both cells types are shown. GO = gene ontology.

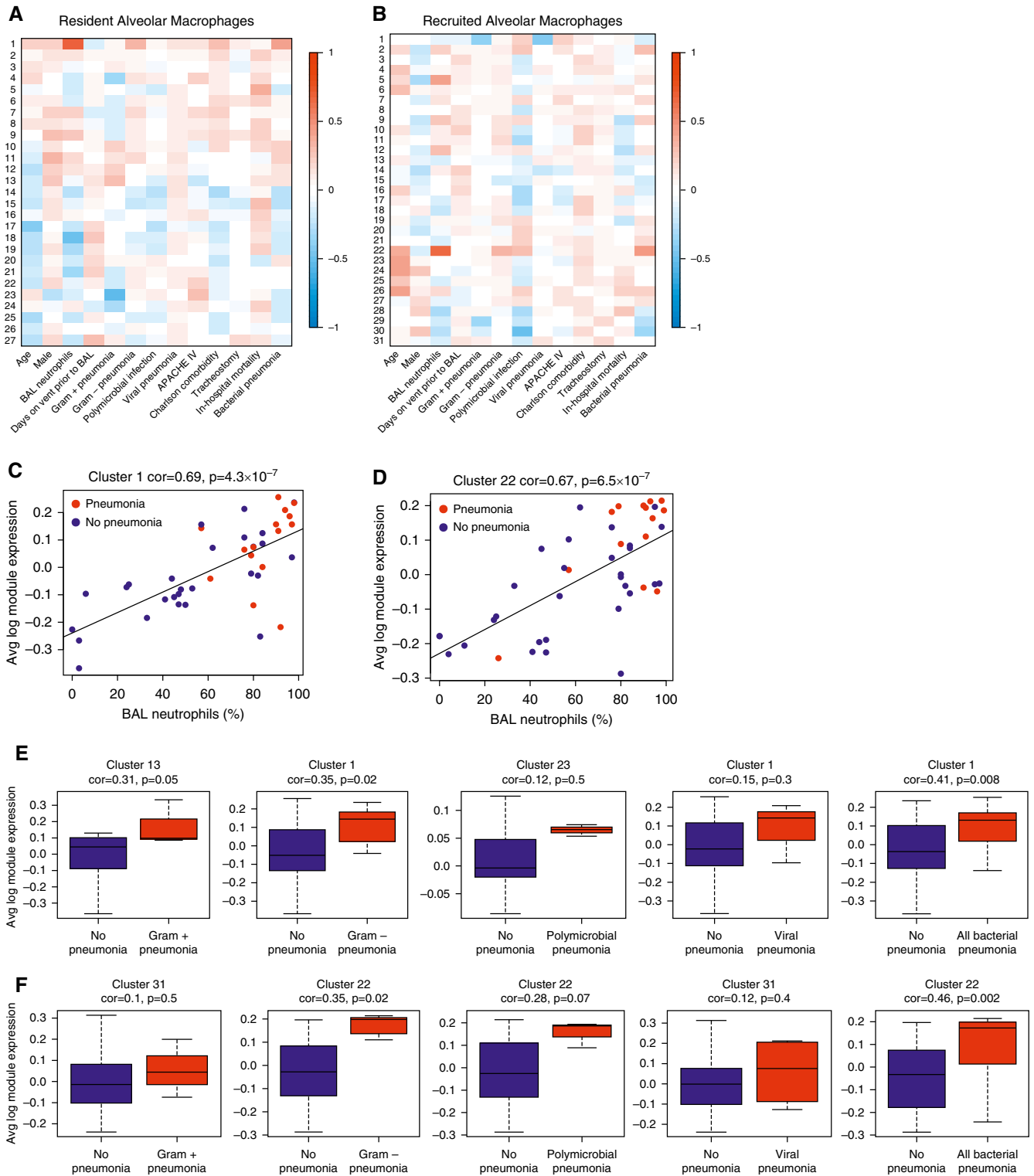


Figure 5. Weighted correlation network analysis (WGCNA) identifies unique gene clusters associated with pneumonia subtypes. (A and B) WGCNA was performed for resident alveolar macrophages (A) and recruited macrophages (B). Each column represents a unique clinical variable. For resident macrophages, row 1 identifies a cluster of 391 genes strongly associated with both BAL neutrophilia and bacterial pneumonia. For recruited macrophages, cluster 22 identifies a cluster of 220 genes strongly associated with both BAL neutrophilia and bacterial pneumonia. (C and D) Average log module expression of these two clusters is plotted against the percentage of neutrophils in BAL fluid. (E and F) Average log module expression of gene clusters with the strongest positive association with pneumonia subtypes. (E) Expression for resident alveolar macrophages; (F) expression for recruited macrophages. APACHE = Acute Physiology and Chronic Health Evaluation; Avg = average; cor = correlation coefficient.

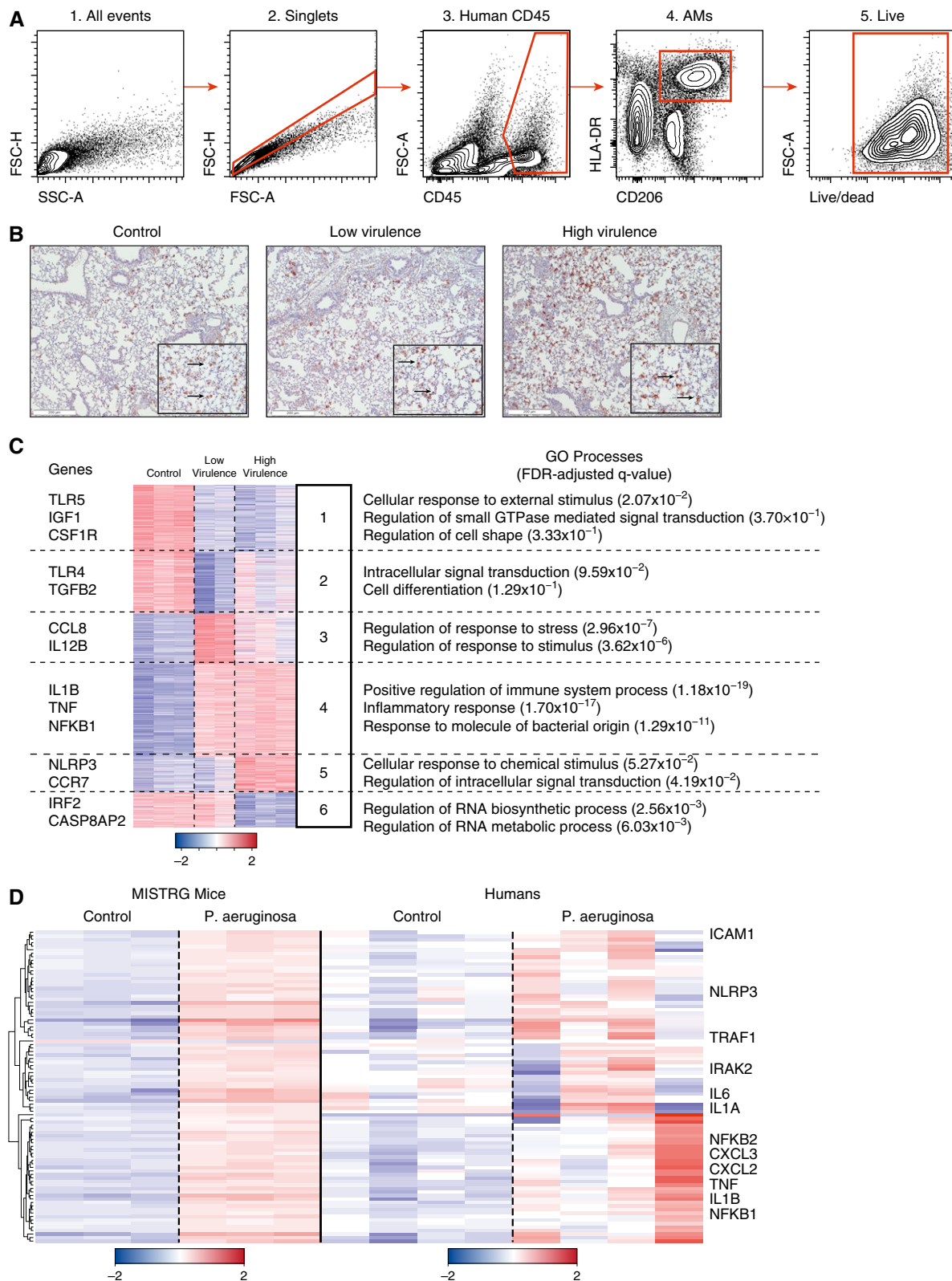


Figure 6. *Pseudomonas aeruginosa* pneumonia produces an identifiable transcriptional signature in alveolar macrophages from humanized (MISTRG) mice. (A) Gating strategy used to identify human alveolar macrophages in humanized mice. (B) Immunohistochemistry for CD206 demonstrates CD206⁺ cells in the alveolar space of humanized mice (black arrows). Representative histology from each experimental group is shown. Scale bars = 200 μm ; insets, scale bars = 100 μm . (C) *k*-means clustering ($k = 6$) of 3,078 differentially expressed genes between all groups. Genes were selected using an

patients with bacterial pneumonia with encouraging operating characteristics despite significant clinical heterogeneity in our study cohort, including a small number of patients infected with the same pathogen. We envision gene signatures identified with this approach being used to inform genes that could be included in PCR-based diagnostic tests—technology that is based on similar chemistry to RNA-Seq.

Although both resident and recruited macrophages exhibited a transcriptional response to bacterial infection, this response differed between cell types. We did not identify any shared differentially expressed genes between resident and recruited macrophages from patients with pneumonia using an FDR-adjusted q value less than 0.05, and only 143 shared genes when using an unadjusted P value less than 0.05. Our results are consistent with animal models describing distinct transcriptional responses to lung injury among macrophage subsets (22, 42). We found that gene expression changes in resident macrophages were more predictive of bacterial pneumonia than changes in recruited macrophages. Although monocyte-derived cells play a prominent role in disease pathogenesis, monocyte-to-macrophage differentiation is associated with dynamic remodeling of more than 60% of the transcriptome (22, 43). It is therefore not surprising that infection-specific gene signatures might be more readily identified in a fully mature cell like the tissue-resident alveolar macrophage.

We concluded by asking whether host response signatures might aid our ability to attribute pneumonia to a specific pathogen. We compared the transcriptomic response of human alveolar macrophages using humanized mice infected with two clinical strains of *P. aeruginosa*. Infection with *P. aeruginosa* was chosen as it is one of the most frequently isolated pathogens in hospital-acquired pneumonia (44, 45). Infection with a virulent strain of *P. aeruginosa* produced a transcriptomic response

distinct from infection with a low virulence strain and from control subjects. We then compared expression of the top 100 differentially expressed genes from this gene set with expression of those same genes in patients with *P. aeruginosa* from our prospective cohort with *P. aeruginosa* pneumonia and in uninfected control subjects. Expression of these genes notably differed between the two patient groups, suggesting that humanized mice might serve as a novel translational tool to inform the identification of pathogen-specific host response signatures in patients.

Our work has several important implications. Diagnostic tests that move beyond pathogen detection and instead identify informative elements of the host response to infection are needed to drive innovation in the diagnosis and management of patients with pneumonia (10). Ideally, a multifaceted assessment of the host response will incorporate molecular characterization of host–pathogen interactions. However, the application of next-generation sequencing technologies to clinical specimens obtained from critically ill patients is challenging and understudied. We demonstrate that RNA-Seq can be used to assess transcriptome-level changes in alveolar macrophages using samples obtained as part of routine clinical care in the ICU. Furthermore, we provide data to suggest that the application of machine learning tools to larger datasets may identify biomarkers that predict causative pathogens. Importantly, we have recently shown that NBBAL is safe and easily performed by nonphysicians (12). Our approach, therefore, could readily be performed serially in informative cohorts, including those infected with high-risk pathogens or after the initiation of a targeted therapy, enabling an unprecedented evaluation of dynamic changes in the alveolar transcriptome. We believe our approach will help answer many of the key questions identified in a recent NHLBI working group report, “Future Research Directions in Pneumonia” (10).

Our study has limitations. Our definition of pneumonia required positive quantitative culture of lower respiratory tract specimens. The clinical utility of such an approach is controversial (18). We considered isolation of oropharyngeal pathogens in lower respiratory culture indicative of true pneumonia if all other clinical criteria were met. Although these bacteria may behave like typical pathogens, their clinical significance remains a source of debate (46). The yield of BAL cultures may have been impacted by antibiotic administration leading to sample misclassification. The operating characteristics of BAL neutrophilia may differ in populations where the pretest probability of pneumonia is lower. Because of sample volume and quality, not all patients in our prospective cohort had sufficient resident and recruited macrophages sorted for RNA sequencing. In addition, the delay from the time of collection to sample processing may have impacted our transcriptome analysis. Finally, although the largest series to date, sample size for our transcriptomic analysis is still too small to reliably generate and validate specific biomarkers.

In conclusion, assessment of alveolar neutrophilia can help to rapidly exclude bacterial pneumonia in critically ill patients, with an NPV approaching 100% when an alveolar neutrophil percentage of less than 50% is paired with a negative lavage fluid gram stain. Resident and recruited macrophages can be isolated from BAL fluid obtained during routine ICU care and used for RNA-Seq analysis. This approach may be useful to identify transcriptional signatures that characterize the host response to bacterial pneumonia. A combination of cellular and molecular analysis of the host and pathogen in BAL fluid shows promise for improving the diagnosis and management of pneumonia. ■

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Figure 6. (Continued). ANOVA approach and a false discovery rate (FDR) q value less than 0.05. (D) Expression of the top 100 differentially expressed genes (identified using an FDR q value) in humanized mice infected with a high-virulence strain of *P. aeruginosa* compared with control subjects was assessed in four patients with *P. aeruginosa* pneumonia and four mechanically ventilated patients without infection. Counts for mice and humans were normalized separately. Representative genes are shown to the right of the figure. Each row represents an individual gene. Each column represents an individual mouse or patient. AM = alveolar macrophage; CD = cluster of differentiation; FSC-A = forward scatter-area; FSC-H = forward scatter-height.

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