Acute Respiratory Distress Syndrome Neutrophils Have a Distinct Phenotype and Are Resistant to Phosphoinositide 3-Kinase Inhibition

Jatinder K. Juss^{1*}, David House², Augustin Amour², Malcolm Begg², Jurgen Herre³, Daniel M. L. Storisteanu¹, Kim Hoenderdos¹, Glyn Bradley⁴, Mark Lennon⁴, Charlotte Summers¹, Edith M. Hessel², Alison Condliffe^{1‡}, and Edwin R. Chilvers^{1‡}

¹Department of Medicine, University of Cambridge, Cambridge, United Kingdom; ²Refractory Respiratory Inflammation Discovery Performance Unit and ⁴Target Sciences, GlaxoSmithKline, Stevenage, United Kingdom; and ³Cambridge University Hospitals NHS Foundation Trust, Cambridge, United Kingdom

Abstract

Rationale: Acute respiratory distress syndrome is refractory to pharmacological intervention. Inappropriate activation of alveolar neutrophils is believed to underpin this disease's complex pathophysiology, yet these cells have been little studied.

Objectives: To examine the functional and transcriptional profiles of patient blood and alveolar neutrophils compared with healthy volunteer cells, and to define their sensitivity to phosphoinositide 3-kinase inhibition.

Methods: Twenty-three ventilated patients underwent bronchoalveolar lavage. Alveolar and blood neutrophil apoptosis, phagocytosis, and adhesion molecules were quantified by flow cytometry, and oxidase responses were quantified by chemiluminescence. Cytokine and transcriptional profiling were used in multiplex and GeneChip arrays.

Measurements and Main Results: Patient blood and alveolar neutrophils were distinct from healthy circulating cells, with increased CD11b and reduced CD62L expression, delayed

constitutive apoptosis, and primed oxidase responses. Incubating control cells with disease bronchoalveolar lavage recapitulated the aberrant functional phenotype, and this could be reversed by phosphoinositide 3-kinase inhibitors. In contrast, the pro-survival phenotype of patient cells was resistant to phosphoinositide 3-kinase inhibition. RNA transcriptomic analysis revealed modified immune, cytoskeletal, and cell death pathways in patient cells, aligning closely to sepsis and burns datasets but not to phosphoinositide 3-kinase signatures.

Conclusions: Acute respiratory distress syndrome blood and alveolar neutrophils display a distinct primed pro-survival profile and transcriptional signature. The enhanced respiratory burst was phosphoinositide 3-kinase-dependent but delayed apoptosis and the altered transcriptional profile were not. These unexpected findings cast doubt over the utility of phosphoinositide 3-kinase inhibition in acute respiratory distress syndrome and highlight the importance of evaluating novel therapeutic strategies in patient-derived cells.

Keywords: acute respiratory distress syndrome; neutrophil function; phosphoinositide 3-kinase

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*Present address: Li Ka Shing Knowledge Institute, St. Michael's Hospital, Toronto, Ontario Canada.

‡These authors contributed equally to this work.

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Correspondence and requests for reprints should be addressed to Edwin R. Chilvers, Ph.D., Sc.D., Department of Medicine, University of Cambridge School of Clinical Medicine, Box 157, Addenbrooke's and Papworth Hospitals, Hills Road, Cambridge CB2 0QQ, UK. E-mail: erc24@cam.ac.uk

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At a Glance Commentary

Scientific Knowledge on the

Subject: Accumulation of polymorphonuclear neutrophils (PMNs) in the lung microvasculature and interstitial and alveolar compartments is a key feature of acute respiratory distress syndrome (ARDS), and inappropriate accumulation and/or activation of alveolar PMNs is proposed to cause local injury by release of oxygen radicals, proteases and neutrophil extracellular traps. Because of the challenges inherent in isolating alveolar PMNs in ARDS, their functional activity and responses to pharmacological agents are largely unknown.

What This Study Adds to the

Field: ARDS alveolar PMNs are phenotypically distinct from their circulating counterparts, and display delayed apoptosis but preserved oxidative burst, phagocytosis, and neutrophil extracellular trap responses. ARDS blood PMNs have an intermediate phenotype between ARDS alveolar PMNs and healthy circulating cells, with a transcriptome showing significant alterations in cell survival and inflammatory pathways. This work improves our understanding of PMN function in ARDS and highlights the importance of studying tissue- and disease-specific cell populations.

The acute respiratory distress syndrome (ARDS) is characterized by diffuse alveolar injury and immune cell infiltration, resulting in intractable hypoxemia (1). Despite the adoption of lung-protective ventilation, mortality remains high (2), and many survivors have long-term physical or neurocognitive sequelae, with fewer than 50% returning to work (2). Management remains largely supportive with optimization of ventilator parameters (3), judicious fluid balance, and treatment of underlying causes; no pharmacological interventions have proven beneficial.

Accumulation of polymorphonuclear neutrophils (PMNs) in the lung microvasculature, interstitial and alveolar compartments is a key feature of ARDS (4),

and an association has been reported between intensity of alveolar neutrophil infiltration and disease severity (5). Inappropriate accumulation and/or activation of PMNs within the alveoli is proposed to cause unrestrained release of oxygen radicals, proteases, and neutrophil extracellular traps (NETs). Due to challenges inherent in isolating alveolar PMNs (^{alv}PMNs), their functional activity in ARDS is largely unknown. Historically, mouse models have been used as surrogates for ^{alv}PMNs (6); however, rodent neutrophils differ markedly from their human counterparts.

Traditionally, PMNs have been viewed as a homogeneous population of short-lived cells with limited transcriptional capacity and a fixed functional repertoire. More recently, concepts of long-lived PMNs, retrograde transendothelial migration, and PMN plasticity have emerged (7–12). Given recent demonstrations that PMNs can modify their transcriptional profile following an inflammatory insult (13), genome-wide transcriptional analysis provides a powerful tool to identify novel targets relevant to altered PMN functions, and it has been successfully applied in asthma, pulmonary arterial hypertension, and ARDS (14); however, studies in ARDS are based on the analysis of total peripheral white blood cells rather than purified neutrophils.

Lung epithelial cells (15) synthesize granulocyte-macrophage colony-stimulating factor (GM-CSF), a cytokine essential for alveolar macrophage function (16, 17) and surfactant homeostasis (18, 19). Conversely, during inflammation, GM-CSF potentiates superoxide anion production (20), promotes PMN survival (21), and is detrimental in models of acute lung injury (22). PMN longevity increases dramatically in ARDS, and some studies have identified GM-CSF as a major prosurvival mediator (23). While the molecular mechanisms governing PMN lifespan in ARDS are incompletely understood, the cytoprotective effect of GM-CSF in PMNs *in vitro* is class I phosphoinositide 3-kinase (PI3K) dependent (21). PI3K inhibition prevents lung tissue edema and leukocyte recruitment in models of ARDS (24), and inhibition of PI3K- γ in a sepsis model reduces end-organ damage (25). Following the early exuberant proinflammatory response in ARDS, patients develop immune paresis, increasing susceptibility to nosocomial infections (26). Recent studies have demonstrated that inhibition of PI3K- δ

may improve PMN responses during this phase (27). These observations have triggered considerable interest in the therapeutic use of PI3K inhibitors in ARDS.

To our knowledge, we present the first comprehensive characterization of purified ARDS blood PMNs (^{blood}PMNs) and ^{alv}PMNs and the first genome-wide transcriptome analysis of purified ARDS ^{blood}PMNs. We show that ARDS ^{alv}PMNs are hypersegmented, with enhanced CD11b and reduced CD62L expression, and display delayed apoptosis but preserved oxidative burst, phagocytosis, and NET responses. ARDS ^{blood}PMNs display an intermediate phenotype, with a transcriptome showing significant alterations in cell survival and inflammatory pathways but little overlap with the PI3K-dependent gene signature. This work improves understanding of PMN function in ARDS and reveals that apoptosis of ARDS neutrophils is resistant to PI3K inhibition. Together, these observations strengthen the case for modulating PMN function in ARDS, but they cast doubt over the utility of PI3K inhibitors in this condition. Some results of these studies have been reported previously in the form of an abstract (28).

Methods

Ethics

All studies complied with the Declaration of Helsinki. Written informed consent was obtained from the legal surrogate of patients with ARDS (UK08/H0306/17). Paired blood samples were obtained simultaneously from age- and sex-matched healthy volunteers (HVs) (UK06/Q0108/281).

Bronchoalveolar Lavage

Patients fulfilling the Berlin criteria for ARDS (29) were recruited from mixed medical-surgical and neurosciences-trauma intensive care units in a U.K. teaching hospital. Exclusion criteria were age younger than 18 years, HIV-positive status, or informed assent could not be obtained. The median tidal volume in the patients with ARDS was 7.64 ml/kg predicted body weight (interquartile range, 6.94–8.64 ml/kg). Patients underwent venipuncture, fiberoptic bronchoscopy (FOB), and bronchoalveolar lavage (BAL) within 48 hours of diagnosis. Sterile isotonic saline (3 $\times$ 50 ml) was instilled into a

subsegmental bronchus. Recovery averaged 90 ml (range, 20–120 ml) and did not differ between patients and HVs. Bronchoalveolar lavage fluid (BALF) was immediately filtered and placed on ice. Control BALF was collected from patients (n = 10) undergoing elective FOB for indications unrelated to infection or ARDS.

PMN Purification

^{alv}PMNs and autologous ^{blood}PMNs were isolated from patients alongside ^{blood}PMNs from age- and sex-matched HVs. ^{alv}PMNs were purified by immunomagnetic negative selection (RoboSep kit; STEMCELL Technologies, Vancouver, BC, Canada) (30) to greater than 90% purity and greater than 98% viability. ^{blood}PMNs were purified over discontinuous plasma Percoll gradients (28).

PMN Morphology

PMNs were classified by nuclear morphology, with the assessor blinded to sample origin. Mature PMNs displayed three or four nuclear lobes connected by heterochromatin filaments. Band PMNs had less condensed chromatin and incompletely segmented nuclei (31), while hypersegmented PMNs possessed at least five lobes.

PMN Activation and Apoptosis

HV and ARDS ^{blood}PMNs and unprocessed BALF were resuspended in phosphate-buffered saline containing 5% bovine serum albumin and protease inhibitor cocktail

(cOmplete Mini, ethylenediaminetetraacetic acid free; Roche Diagnostics, Indianapolis, IN). Samples were stained with CD62L-allophycocyanin (clone 559772; BD Pharmingen, San Jose, CA), CD11b-fluorescein isothiocyanate (clone IM0530; Beckman Coulter, Brea, CA), or isotype-matched controls. PMN apoptosis was assessed after 20 hours by flow cytometry using fluorescein isothiocyanate-labeled Annexin V/propidium iodide (BD Pharmingen).

PMN Oxidative Burst

Neutrophils (5×10^6 /ml) were primed with tumor necrosis factor (TNF)- α (R&D Systems, Minneapolis, MN), GM-CSF, control BALF, or ARDS BALF at 37°C for 30 minutes. The oxidative burst in response to formylmethionylleucylphenylalanine (FMLP) (100 nM), zymosan, or heat-killed *Streptococcus pneumoniae* (serum opsonized, five to seven particles per PMN) was assessed by luminol-dependent chemiluminescence (30).

Quantification of Inflammatory Mediators

BALF and serum mediators were measured by ELISA (LPS; Kamiya Biomedical Co., Seattle, WA), surviving, and leukotriene B₄ (R&D Systems) or using the V-PLEX Human Biomarker 40-Plex Kit and the Human MMP 3-Plex Ultra-Sensitive Kit (both from Meso Scale Discovery, Rockville, MD). Where stated, BALF

samples were corrected to the total protein concentration (Pierce bicinchoninic acid protein assay; Thermo Scientific, Rockford, IL).

PMN Phagocytosis

PMN phagocytic capacity was assessed using 1 mg/ml pHrodo Red *Staphylococcus aureus* Bioparticles (Life Technologies, Carlsbad, CA). Internalization was verified by live confocal imaging.

NET Formation

^{blood}PMNs and ^{alv}PMNs (1×10^6 /ml) incubated with SYTOX Green (5 μ M; Life Technologies) were seeded onto 96-well optical microplates (BD Biosciences, San Jose, CA). NET formation was quantitated by hourly fluorescence measurements and verified by fluorescence microscopy using rabbit antihistone H3 (Ab5103; Abcam, Cambridge, UK).

Neutrophil Cytoskeletal Remodeling

Freshly isolated PMNs (1×10^6 /ml) were fixed (4% paraformaldehyde), permeabilized (0.5% Triton X-100), and stained with antineutrophil elastase (1:1,000; Santa Cruz Biotechnology, Dallas, TX) and rhodamine phalloidin (1:200; Invitrogen, Carlsbad, CA).

Genome-Wide Transcriptomic Changes

Genome-wide transcriptomic changes were assessed in paired ^{blood}PMNs from

Table 1. Demographics and Clinical Characteristics of Patients with Acute Respiratory Distress Syndrome

	ARDS		
	Mild	Moderate	Severe
Number of patients	8	11	4
Age, yr	49 $\pm$ 16.3	62.7 $\pm$ 11.1	48.3 $\pm$ 21.0
Sex ratio, male:female	7:1	5:6	3:1
Patient mortality, n	2	7	2
PaO ₂ , mm Hg	92.2 $\pm$ 17.6	82.4 $\pm$ 16.8	68.4 $\pm$ 2.3
PaO ₂ /FiO ₂ , mm Hg	246.1 $\pm$ 18.9	139.5 $\pm$ 18.8	90.0 $\pm$ 5.7
PEEP, cm H ₂ O	8.9 $\pm$ 2.1	8.9 $\pm$ 2.6	8.5 $\pm$ 1.9
Blood WBC count	11.1 $\pm$ 10.1	10.8 $\pm$ 8.0	12.9 $\pm$ 8.4
Blood PMN count	8.4 $\pm$ 7.2	9.7 $\pm$ 7.9	11.4 $\pm$ 7.5
PMNs in BALF, %	69.7 $\pm$ 2 (n = 6)	73.7 $\pm$ 11.1 (n = 10)	59.8 $\pm$ 2 (n = 3)
Etiology of ARDS	Community-acquired pneumonia, neutropenic sepsis, freshwater drowning, liver failure post-transplant donation, ethylene glycol poisoning	Community-acquired pneumonia, aspiration pneumonia, neutropenic sepsis	Community-acquired pneumonia, aspiration pneumonia, neutropenic sepsis

Definition of abbreviations: ARDS = acute respiratory distress syndrome; BALF = bronchoalveolar lavage fluid; PEEP = positive end-expiratory pressure; PMN = polymorphonuclear neutrophil; WBC = white blood cell. Data are presented as mean $\pm$ SD unless otherwise indicated.

12 consecutively recruited patients with ARDS who were representative of the full patient cohort in terms of age, sex, and ARDS severity and causation, as well as from 12 HVs. Further studies were undertaken in the following groups of HV^{blood}PMNs (n = 10 per group):

(1) 0 hours, vehicle control; (2) 6 hours, vehicle control; (3) 6 hours, recombinant human GM-CSF (rhGM-CSF) (1 ng/ml); (4) 6 hours, pan-PI3K inhibitor ZSTK474 (10 μ M); and (5) 6 hours, rhGM-CSF plus ZSTK474.

Complementary DNA (cDNA) prepared from 2.5 ng of RNA using the WT-Ovation Pico RNA Amplification

System (NuGEN Technologies, San Carlos, CA) was fragmented and labeled using the FL-Ovation cDNA Biotin Module V2 (NuGEN). Labeled cDNA was hybridized onto GeneChip Human Genome U133 Plus 2.0 oligonucleotide arrays (Affymetrix, Santa Clara, CA). Raw data (see online supplement) were normalized using the robust multiarray average method (32) and quality checked in R/Bioconductor. A linear model was fitted to normalized data for each probe set, and a *post hoc* test (Fisher's least significant difference) generated fold changes and *P* values. Probes were identified as significant if their fold change

was greater than 1.5 and the *P* value was less than 0.05, and they were mapped to pathways using Ingenuity Pathway Analysis software (QIAGEN, Redwood City, CA). The NextBio analysis platform (Illumina, San Diego, CA) was used to compare our ARDS data with (preanalyzed) publicly available transcriptomic data.

Statistical Analysis

For each dataset analyzed, an appropriate linear mixed model was fitted. When required, the data were logarithmically transformed to meet the assumptions of the analysis (i.e., normally distributed

Figure 1. Circulating and alveolar neutrophils from patients with acute respiratory distress syndrome (ARDS) are phenotypically distinct from healthy volunteer (HV) neutrophils. (A) Morphology of freshly purified polymorphonuclear neutrophils (PMNs) from HVs in comparison with ARDS blood PMNs (bloodPMNs) and alveolar PMNs (alvPMNs) was assessed. Representative photomicrographs of cytopsins ($\times 100$ original magnification; modified Wright stain) of HV^{blood}PMNs and patient^{alv}PMNs and^{blood}ARDS PMNs isolated in parallel (n = 19) are shown. Classical PMNs have three or four nuclear lobes connected by filaments of dense heterochromatin; band PMNs (red arrow) contain a curved nucleus that is not completely segmented into lobes; and hypersegmented PMNs (black arrow) have nuclei comprised of five or more lobes. ARDS bronchoalveolar lavage fluid (BALF) contains abundant hypersegmented PMNs with up to 12 nuclear lobes (inset). PMN subsets present in the blood (HV, n = 19; ARDS, n = 19) and ARDS BALF (n = 11) were quantitated morphologically by light microscopy (right panel). The PMN percentage subsets were analyzed on the log₁₀ scale using a linear fixed effects model (****P* < 0.0001 for hypersegmented cells; ns = not significant). (B) HV^{blood}PMNs and autologous ARDS^{alv}PMNs and^{blood}PMNs stained for F-actin polarization (rhodamine phalloidin, green) and elastase (red), with nuclei depicted in blue (4',6-diamidino-2-phenylindole). Representative immunofluorescence confocal photomicrographs (n = 3; $\times 40$ original magnification) show that ARDS^{alv}PMNs and^{blood}PMNs display a prominent circumferential F-actin ring (white arrows). (C) PMN cell surface CD62L-fluorescein isothiocyanate and CD11b-allophycocyanin expression of freshly isolated HV^{blood}PMNs, autologous ARDS^{alv}PMNs, and^{blood}PMNs assessed by flow cytometry. Results are representative of three independent experiments. The CD11b expression (mean fluorescence intensity [MFI] corrected for isotype control readings) for HV blood neutrophils was 11.7 ± 0.9 , that of ARDS blood neutrophils was 74 ± 6 , and that of ARDS BALF neutrophils 427 ± 50 . The CD62L expression (MFI corrected for isotype control readings) for HV blood neutrophils was 114 ± 12 , that of ARDS blood neutrophils was 8.4 ± 0.6 , and that of ARDS BALF neutrophils was 8.7 ± 1.9 .

errors and homogeneity of variance). Correction for false discovery rates in the transcriptional and cytokine analysis was done according to the method of Benjamini and Hochberg (33). The analyses were conducted in SAS version 9.3 software (SAS Institute, Cary, NC). The results are presented as means $\pm$ SEM of independent experiments, with $P < 0.05$ considered statistically significant. Full details of the number of patients and HVs included in

each assay are provided in Figure E1 in the online supplement.

Results

ARDS Patient Characteristics

Twenty-three mechanically ventilated patients fulfilling the 2011 Berlin definition for ARDS were recruited. Their clinical, demographic, and

physiological characteristics are outlined in Table 1. Standardized ventilator strategies in accordance with the ARDS Network low tidal volume protocol were employed. At sample collection, 4 of 23 patients had severe ARDS ($\text{PaO}_2/\text{FiO}_2$ ratio, ≤ 100 mm Hg), 11 of 23 had moderate ARDS ($\text{PaO}_2/\text{FiO}_2$ ratio, 101–200 mm Hg), and 8 of 23 had mild ARDS ($\text{PaO}_2/\text{FiO}_2$ ratio, 201–300 mm Hg). Sepsis and pneumonia were the

Figure 2. Circulating and alveolar neutrophils from patients with acute respiratory distress syndrome (ARDS) exhibit delayed apoptosis and heterogeneous nicotinamide adenine dinucleotide phosphate oxidase responses. (A) Healthy volunteer (HV) blood polymorphonuclear neutrophils ($5 \times 10^6/\text{ml}$) were cultured in Iscove's modified Dulbecco's medium with 10% autologous serum. Apoptosis was quantitated by flow cytometry following Annexin V (AnV) staining after 20 hours in culture. The data were analyzed using a linear fixed effects model (** $P < 0.001$; *** $P < 0.0001$). (B) Representative kinetic profile of the neutrophil oxidative burst. Freshly purified HV ^{blood}PMNs (unprimed, solid squares; primed with recombinant human tumor necrosis factor [TNF]- α [10 ng/ml], open squares), autologous ARDS alveolar PMNs (^{alv}PMNs) (solid circles), and ^{blood}PMNs (open circles) were incubated with luminol and horseradish peroxidase in a 96-well luminometer plate, and formylmethionyleucylphenylalanine (FMLP) (100 nM) was added via the injection port of a Centro LB 960 luminometer (Berthold Technologies, Bad Wildbad, Germany). Light emission (relative light units) was recorded at 6-second intervals over 10 minutes. (C) The oxidative response in freshly isolated unprimed and recombinant human TNF- α -primed HV blood following stimulation with FMLP is expressed as the relative peak height to the rTNF- α -primed response in HV ^{blood}PMNs. (D) Peak height of the neutrophil oxidative response in freshly isolated autologous ARDS ^{alv}PMNs and ^{blood}PMNs normalized to the HV blood PMN response (i) to FMLP (100 nM), (ii) to serum-opsonized zymosan (five to seven particles per PMN), and (iii) to serum-opsonized, heat-killed *Streptococcus pneumoniae* (five to seven particles per PMN).

commonest precipitating insults. Thirteen of 23 patients survived to discharge. All patients underwent FOB within 48 hours of diagnosis. PMNs constituted $69.7 \pm 4.2\%$ of the differential leukocyte count in ARDS BALF ($6.5 \pm 3.2\%$ in control BALF) (Table 1). PMN abundance in BALF did not correlate with initial ARDS severity, abnormalities in gas

exchange, or BALF protein concentration (data not shown).

ARDS PMNs Are Phenotypically Distinct

Comparison of purified HV^{blood}PMNs, ARDS^{blood}PMNs, and alvPMNs revealed striking differences in cell morphology. While HV^{blood}PMNs had few

hypersegmented PMNs, alvARDS PMNs displayed abundant hypersegmented nuclei and cytoplasmic vacuolation (Figure 1A); hypersegmented PMNs were not identified in control BALF. Immature “band” PMNs were also more common in ARDS^{blood}PMNs and alvPMNs than in control BALF (Figure 1A).

Figure 3. Circulating and alveolar neutrophils from patients with acute respiratory distress syndrome (ARDS) exhibit preserved phagocytic capacity and heterogeneous neutrophil extracellular trap (NET) responses. (A) Analysis of phagocytosis by freshly isolated polymorphonuclear neutrophils (PMNs). Representative immunofluorescence confocal photomicrographs ($n = 5$ experiments) taken after 1-hour incubation demonstrate internalized pHrodo-conjugated *Staphylococcus aureus* (red) and PMN nuclei counterstained with 4',6-diamidino-2-phenylindole (blue). (B) PMN phagocytosis was quantitated by flow cytometry with fluorescence excitation set at 530 nm and emission set at 590 nm. These data were analyzed using a linear fixed effects model (ns [not significant], $P > 0.05$). (C) NET formation by freshly isolated autologous blood and alveolar ARDS PMNs and healthy volunteer (HV) PMNs was assessed following treatment with phorbol myristate acetate (PMA) (20 nM) or vehicle control. Representative fluorescence photomicrographs of NETs ($n = 4$) taken at $\times 63$ original magnification are shown. Unmerged images (lower panels) stained for extracellular DNA scaffold (SYTOX, green) and citrullinated histones (CitHis, red). The precise overlap of these two colors in merged images generates the orange representing NETs in the PMA-treated cells. No NETs are visible in the control samples. (D) The kinetics of NET formation was assessed over 4 hours by measuring total fluorescence using a VICTOR³ Multilabel Reader using Wallac 1420 Workstation v3.00 software (both from PerkinElmer, Waltham, MA) and subtracting baseline fluorescence. Scale bars represent 10 μm .

PMN activation status was assessed by confocal imaging of F-actin and cell surface staining of CD62L (L-selectin) and CD11b (Mac1). Prominent circumferential F-actin fluorescence was observed in a substantial proportion of the ARDS ^{blood}PMNs compared with HV ^{blood}PMNs (Figure 1B). The profile of surface receptor expression (upregulation of CD11b and downregulation of CD62L [34, 35]) on both ARDS ^{blood}PMNs and ^{alv}PMNs was consistent with a primed and/or activated phenotype (Figure 1C).

ARDS Blood and Alveolar PMNs Show Delayed Apoptosis and Primed NADPH Oxidase Responses

Consistent with a previous report (23), we demonstrate that, after 20-hour *ex vivo* incubation, ARDS ^{alv}PMNs and ^{blood}PMNs demonstrated a significantly reduced number of apoptotic cells ($28.5 \pm 19.2\%$ and $42.7 \pm 23\%$ [percentage of apoptosis $\pm$ SEM],

respectively) compared with PMNs isolated from HV blood ($69.2 \pm 12\%$) (Figure 2A). The magnitude of the survival response exhibited by ARDS PMNs was equivalent ($28.6 \pm 10\%$) to the cytoprotective effect conferred by incubating HV ^{blood}PMNs with a maximally effective concentration of rhGM-CSF (1 ng/ml) (data not shown).

We next compared the ability of ARDS ^{alv}PMNs and ^{blood}PMNs to mount an oxidative burst in response to FMLP, opsonized zymosan, and *S. pneumoniae* (Figures 2B–2D) (36). In contrast to unprimed HV ^{blood}PMNs, which displayed minimal reactive oxygen species (ROS) generation to FMLP (Figures 2B and 2C), ^{alv}PMNs and ^{blood}PMNs from patients with ARDS displayed robust ROS generation to all three stimuli, which in certain individuals exceeded those of TNF- α -primed HV ^{blood}PMNs (Figure 2D). These data indicate basal priming and preserved NADPH oxidase responses of

ARDS ^{alv}PMNs and ^{blood}PMNs, challenging the notion that inflammatory PMNs become “exhausted” at peripheral sites.

ARDS Blood and Alveolar PMNs Have Preserved Capacity for Phagocytosis and NET Formation

Previous investigators have identified a defect in the phagocytic and microbicidal activity of neutrophils from patients with ARDS (37). However, in our cohort, flow cytometry and confocal microscopy demonstrated that the capacity of ARDS ^{alv}PMNs and ^{blood}PMNs to phagocytose pHrodo Red-labeled *S. aureus* was fully preserved (Figures 3A–3B). This assay, supported by live-cell imaging, is based on differential fluorescence of this bioparticle in an acidic environment, ensuring that only organisms within functional phagosomes are detected.

In addition to phagocytosis and the oxidative burst, PMNs deploy NETs

Figure 4. Heat map of the inflammatory markers in the serum of healthy volunteers (HVT) and patients with acute respiratory distress syndrome (ARDS). Inflammatory mediators in serum were measured using either an ELISA kit or a Meso Scale Discovery electrochemical luminescence multiplex immunoassay kit. In the heat map, each row is a different cytokine and each column is a different patient. The coloring represents the abundance of the inflammatory marker measured. The lowest abundance measured is presented in *bright green* and the highest in *bright red*. To assess the mean difference in abundance between the disease groups, a linear mixed model was fitted to the data with disease as a fixed effect and the donor pairing as a random effect. The heat maps and dendrogram (variable tree) were obtained from a hierarchical clustering of the cytokines using complete linkage. The variable tree to the left of the heat map shows how the cytokines cluster together based on their Pearson's correlation (i.e., the more correlated two cytokines are, the closer they are in the branches of the dendrogram). The asterisks on the plot represent the false discovery rate (FDR)-adjusted P value of the disease effect in this model (*FDR $P < 0.05$; **FDR $P < 0.001$; ***FDR $P < 0.0001$).

to facilitate pathogen clearance. NETs are composed principally of a DNA scaffold decorated with antimicrobial granule proteins, which acts as a mesh to immobilize pathogens (Figure 3C). In response to phorbol myristate acetate (Figures 3C and 3D) or pyocyanin (data

not shown), ARDS ^{alv}PMNs and ^{blood}PMNs displayed a similar capacity for NET production to that of HV ^{blood}PMNs. Collectively, our results demonstrate preservation of the antimicrobial functions of ARDS ^{alv}PMNs and ^{blood}PMNs.

Defining the Impact of ARDS on Serum/BALF Cytokine Profiles and the Transcriptional Signatures of Blood PMNs

To address whether factors present in the serum or BALF in patients with ARDS could account for the primed

A

Figure 5. Genes altered greater than fivefold in acute respiratory distress syndrome (ARDS) blood neutrophils compared with healthy volunteer (HV) blood neutrophils. (A) Negative values indicate a decrease in relative gene expression, whereas positive values indicate an increase in relative gene expression.

B

Figure 5. (Continued). All $P < 0.05$ ($n = 12$ for ARDS and HV blood polymorphonuclear neutrophils [PMNs]). (B) Diagrammatic representation of gene transcript changes between ARDS and HV blood PMNs and the overlap in the ARDS transcript signature with pediatric early stage burn data (38).

and/or prosurvival PMN phenotype, a series of multiplex ELISAs and bioassays were used to characterize the cytokine and growth factor profiles ($n = 18$). As shown in Figure 4 and Figure E2, a consistent profile of increased acute-phase markers (e.g., C-reactive protein, serum amyloid A) and inflammatory cytokines (e.g., TNF- α , thymus and activation-regulated chemokine, monocyte chemoattractant protein 1, IL-8, and IL-6) was observed in ARDS serum and BALF compared with HV samples. By contrast, when BALF samples were corrected for total protein concentration, only C-reactive protein, IL-6, and monocyte chemoattractant protein 1 levels were significantly higher in ARDS samples than in control samples (Figure E2). Of note, at the single time point sampled, GM-CSF was quantifiable in only 5 of 23 ARDS BALF samples (lower limit of quantification, 7.6 pg/ml).

RNA transcriptomic analysis comparing freshly isolated ARDS blood PMNs with HV blood PMNs revealed a total of 1,319 altered genes (using cutoffs of fold change >1.5 and $P < 0.05$; top-ranked up- and downregulated transcripts are shown in Figure 5A; a full list of all 1,319 genes and their relative fold changes is provided in Table E3). Using the NextBio analysis platform (which recognized 1,282 of the 1,319 differentially expressed genes), we compared these changes with publicly available datasets, finding a striking similarity to data in leukocytes from patients with severe burns or sepsis (38, 39); not only was there a strong overlap in gene changes, but also the direction of change correlated almost completely (Figure 5B). Ingenuity Pathway Analysis revealed a significant increase in pathways associated with the immune response, cytoskeletal remodeling, and mucin production, as well as significant decreases in cell death and/or apoptosis

pathways, consistent with the neutrophil phenotype observed (Figure E3). Of note, of the 1,319 observed transcript changes, only 216 were differentially expressed in the same direction compared with HV blood PMNs treated *ex vivo* with GM-CSF (Figure E4). The data discussed in this publication have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) and are accessible through GEO series accession number GSE76293 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE76293>).

Influence of the Local Airway Environment on the ARDS PMN Phenotype

To establish the significance of the alveolar inflammatory environment, we sought to induce a phenotypic switch using ARDS BALF. Incubating HV blood PMNs with Iscove's modified Dulbecco's medium with 10% autologous serum containing ARDS

BALF (50:50 vol/vol) reduced the extent of apoptosis observed *ex vivo* at 20 hours ($37.4 \pm 21.7\%$; 50:50 vol/vol control BALF, $70.5 \pm 12.4\%$) (Figure 6A). Furthermore, treatment with ARDS BALF (50:50 vol/vol) for 30 minutes enhanced FMLP-induced ROS production in HV ^{blood}PMNs (Figure 6B) to a level comparable to optimally TNF- α - or GM-CSF-primed HV ^{blood}PMNs (not shown), while control BALF had little effect. Thus, ARDS BALF supernatant recapitulated the prosurvival, primed NADPH oxidase signature seen in ARDS ^{blood/alv}PMNs.

Potential Role of PI3K Inhibition as a Strategy to Modulate ARDS Blood and Alveolar PMN Behavior

A key objective of this study was to define the sensitivity of inflammatory PMNs to PI3K inhibition, since this pathway is pivotal in neutrophil survival, priming and/or activation, and ROS production (30). First, we confirmed that a pan-PI3K inhibitor, ZSTK474 (10 μ M) (40), and to a lesser extent the p38 mitogen-activated protein kinase inhibitor SB741445 (10 μ M), blocked GM-CSF-induced PMN survival in HV ^{blood}PMNs *in vitro* (Figure 7A). ZSTK474 also blocked the survival effect of ARDS BALF supernatant on HV ^{blood}PMNs (Figure 7B). However, neither compound restored normal neutrophil apoptosis in ARDS ^{blood}PMNs (Figure 7C), implying that the aberrant disease-associated neutrophil survival is either irreversible or operates through a PI3K-independent pathway. Given that even the delayed addition of ZSTK474 to GM-CSF-treated HV ^{blood}PMNs retained effectiveness in overcoming the prosurvival effect of this cytokine (data not shown), the involvement of a PI3K-independent pathway seems most likely. This conclusion is supported by the minimal overlap we observed between the transcriptomal signatures seen in the ARDS ^{blood}PMNs and those seen in HV ^{blood}PMNs treated with ZSTK474 (Figure E5). In contrast, ROS production by PMNs from HVs and patients with ARDS was completely abrogated by ZSTK474 (Figure 7D).

Discussion

Isoform-selective PI3K inhibitors have been proposed as antiinflammatory agents

Figure 6. Treatment of healthy volunteer (HV) blood neutrophils with bronchoalveolar lavage fluid (BALF) from patients with acute respiratory distress syndrome (ARDS) replicates the prosurvival and primed neutrophil phenotype. (A) HV polymorphonuclear neutrophils (PMNs) were incubated in Iscove's modified Dulbecco's medium with 10% autologous serum containing either 50:50 vol/vol control BALF or ARDS BALF, and apoptosis was assessed at 20 hours (HRS) by flow cytometry following Annexin V (AnV) staining. These data were analyzed using a linear mixed effects model with the HV donor fitted as a random effect (not significant [ns], $P > 0.05$; $***P < 0.0001$). (B) Freshly isolated HV PMNs were treated with 50:50 vol/vol control BALF or ARDS BALF for 30 minutes at 37°C before stimulation with formylmethionyleucylphenylalanine (100 nM). Chemiluminescence was recorded using a Centro LB 960 luminometer and expressed graphically as the absolute peak height in relative light units. These data were analyzed on the $\log_{10}$ scale using a linear mixed effects model with the HV donor fitted as a random effect (ns, $P > 0.05$; $***P < 0.0001$).

in diseases such as ARDS (41). Hence, it is important to study their efficacy in patient-derived cells. ARDS ^{alv}PMNs have been little studied due to the difficulty of obtaining these cells from acutely unwell patients. Using purified blood and alveolar neutrophils from 23 patients with ARDS, we demonstrate a stepwise change from HV ^{blood}PMNs through ARDS ^{blood}PMNs to ARDS ^{alv}PMNs. ARDS ^{alv}PMNs, and to a lesser extent ARDS ^{blood}PMNs, were distinct from HV ^{blood}PMNs, with hypersegmented nuclei, increased CD11b expression, prolonged survival, and primed NADPH oxidase responses. Surprisingly, while the respiratory burst remained fully sensitive to PI3K inhibition, the prosurvival phenotype was not reversed by this strategy.

Few previous studies have included assessment of the characteristics of paired circulating and postmigration inflammatory tissue neutrophils. The hypersegmented CD11b^{high}/CD62L^{low} cells with enhanced oxidative capacity we identified in ARDS ^{blood} and ARDS

^{alv}PMNs are reminiscent of circulating neutrophils isolated following endotoxin challenge (42, 43). These latter cells were immunosuppressive, inhibiting T-cell proliferation by release of hydrogen peroxide at the neutrophil-T-cell interface. Increased nuclear segmentation and oxidative potential has also been observed in tumor-associated neutrophils (44), associated with increased antitumor activity. Prolonged survival of ARDS ^{alv}PMNs has been reported previously and attributed to GM-CSF and/or granulocyte colony-stimulating factor in BALF (23); however, in contrast to Matute-Bello and colleagues, we did not observe significantly elevated levels of these cytokines, perhaps related to disease heterogeneity and differences in sampling time. Delayed apoptosis has been measured in neutrophils recruited to skin chambers versus paired circulating neutrophils, but synovial fluid-derived PMNs from patients with rheumatoid arthritis exhibited normal apoptosis (45). These differences correlated with

Figure 7. Effects of phosphoinositide 3-kinase (PI3K) and p38 mitogen-activated protein kinase (MAPK) inhibition on healthy blood and acute respiratory distress syndrome (ARDS) neutrophils. (A) Healthy volunteer (HV) blood polymorphonuclear neutrophils (PMNs) were preincubated with either 0.1% dimethyl sulfoxide vehicle control, p38 MAPK inhibitor (SB741445 [10 μ M]) or pan-class I PI3K inhibitor (ZSTK474 [10 μ M]) for 20 minutes and then treated with recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF [1 ng/ml]). Apoptosis was quantitated after 20 hours (HRS) in culture by flow cytometry following Annexin V (AnV) and propidium iodide (PI) staining. These data were analyzed using a linear mixed effects model with the HV donor fitted as a random effect ($***P < 0.0001$). (B) HV blood PMNs were preincubated with the indicated inhibitors for 20 minutes before culture in 50:50 vol/vol ARDS bronchoalveolar lavage fluid. Apoptosis was quantitated after 20 hours in culture by flow cytometry following AnV and PI staining. These data were analyzed using a linear mixed effects model with the HV donor fitted as a random effect (not significant [ns], $P > 0.05$; $**P < 0.001$). (C) ARDS blood PMNs were incubated with SB741445 or ZSTK474, and apoptosis was quantitated by flow cytometry following AnV and PI staining. These data were analyzed using a linear mixed effects model with the HV donor fitted as a random effect (ns, $P > 0.05$; $*P < 0.05$). (D) ARDS blood and alveolar PMNs were preincubated with ZSTK474 (10 μ M) for 30 minutes at 37°C before stimulation with formylmethionylleucylphenylalanine (100 nM). Chemiluminescence was recorded using a Centro LB 960 luminometer and expressed graphically as the absolute peak height in relative light units. Data were analyzed on the log₁₀ scale using a linear mixed effects model with the HV donor fitted as a random effect ($*P < 0.05$; $***P < 0.0001$). TNF = tumor necrosis factor.

local IL-1 β levels, but IL-1 β in the ARDS BALF in our study was not significantly elevated. The variable functional capacity of neutrophils from different locations underscores the need to explore the efficacy of potential therapeutic agents in disease-relevant cell populations.

We observed a proinflammatory cytokine profile in the blood of patients with ARDS, including several established priming agents. In our present study, ARDS^{blood} and ARDS^{alv} PMNs were functionally primed, and such cells have previously been implicated in lung injury (46, 47). We previously

demonstrated that the pulmonary capillary bed can trap and “de-prime” neutrophils and that this mechanism may fail in ARDS, augmenting the circulating pool of these potentially injurious cells (48). Additional priming signals may be imparted during vascular transmigration (49), and ARDS BALF also primed the oxidative burst of HV^{blood} PMNs. Thus, a number of different factors may contribute to the pooling of primed neutrophils within the alveolar environment in ARDS.

ARDS^{blood} PMNs, and in particular ARDS^{alv} PMNs, survived longer than HV^{blood} PMNs during *ex vivo* culture. This prosurvival phenotype was recapitulated by incubating HV^{blood} PMNs with ARDS BALF, implying that the enhanced longevity of these cells results at least in part from local exposure to mediators. However, while the pan-PI3K inhibitor ZSTK474 did not reduce the lifespan of ARDS^{blood} PMNs, it did reverse the prosurvival effects of both GM-CSF and BALF on HV^{blood} PMNs in culture, suggesting that the complex cytokine environment in BALF is not the only factor conferring PI3K resistance. It is possible that the duration of exposure to prosurvival mediators *in vivo* before inhibitor exposure is relevant and that survival signals imparted during transmigration will likewise have been entrained before PI3K inhibition. Finally, hypoxia may impart additional signals that are also relatively PI3K resistant, and hypoxia-inducible factor–dependent signaling was upregulated (ranked 14th in the pathways changed in this setting; *see* Table E3). Together with the limited overlap between the ZSTK474^{blood} PMN or GM-CSF^{blood} PMN transcriptomes and the ARDS^{blood} PMN signature, our results suggest that targeting of PI3K during ARDS, while suppressing the damaging ROS formation, would not enhance cell clearance via apoptotic pathways.

We further interrogated the activation state of ARDS^{blood} PMNs by undertaking the first reported transcriptomic analysis of purified peripheral^{blood} PMNs from patients with ARDS. Our data revealed remarkable overlap between the transcriptomic profile of ARDS^{blood} PMNs and those published for mixed leukocytes in burns (*see* Figure 4B) and sepsis cohorts (33, 36). The top five canonical pathways

identified in the ARDS blood neutrophil gene signature were the glucocorticoid, IL-4, p38 mitogen-activated protein kinase, antigen presentation, and CDC52 pathways. These were also within the top five pathways identified in the previous burns and sepsis cohorts using mixed leukocytes (38, 39). This suggests that, despite their heterogeneity, there is a strong commonality in a range of acute severe inflammatory disorders. This also provides possible directions for novel therapeutic interventions aimed at, for example, the IL-4 receptor or p38 pathways.

In this study, we sought to characterize the functional and transcriptional profile of PMNs isolated from the blood and

airways of patients with ARDS. Although our study captured only 23 patients at a single time point, our data add considerably to knowledge of ^{alv}PMN and ^{blood}PMN function and signaling profiles in ARDS. They challenge data derived from both animal models and healthy cells, with a marked primed and prosurvival phenotype, the latter recalcitrant to PI3K inhibition. We conclude that intervention with a PI3K inhibitor in these patients is unlikely to be an effective therapeutic strategy, since it will impair PMN bactericidal function without facilitating inflammation resolution. Our findings highlight the importance of working with patient-derived cells, particularly for

biomedical research into novel treatments for ARDS. ■

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