ORIGINAL ARTICLE

Increased Alternative Complement Pathway Function and Improved Survival during Critical Illness

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Abstract

Rationale: Complement is crucial for host defense but may also drive dysregulated inflammation. There is limited understanding of alternative complement function, which can amplify all complement activity, during critical illness.

Objectives: We examined the function and key components of the alternative complement pathway in a series of critically ill patients and in a mouse pneumonia model.

Methods: Total classical (CH50) and alternative complement (AH50) function were quantified in serum from 321 prospectively enrolled critically ill patients and compared with clinical outcomes. Alternative pathway (AP) regulatory factors were quantified by ELISA ($n = 181$) and examined via transcriptomics data from external cohorts. Wild-type, $Cfb^{-/-}$, and $C3^{-/-}$ mice were infected intratracheally with *Klebsiella pneumoniae* (KP) and assessed for extrapulmonary dissemination.

Measurements and Main Results: AH50 greater than or equal to median, but not CH50 greater than or equal to median, was associated with decreased 30-day mortality (adjusted odds ratio

[OR], 0.53 [95% confidence interval (CI), 0.31–0.91]), independent of chronic liver disease. One-year survival was improved in patients with AH50 greater than or equal to median (adjusted hazard ratio = 0.59 [95% CI, 0.41–0.87]). Patients with elevated AH50 had increased levels of AP factors B, H, and properdin, and fewer showed a "hyperinflammatory" subphenotype (OR, 0.30 [95% CI, 0.18–0.49]). Increased expression of proximal AP genes was associated with improved survival in two external cohorts. AH50 greater than or equal to median was associated with fewer bloodstream infections (OR, 0.67 [95% CI, 0.45–0.98]). Conversely, depletion of AP factors, or AH50 less than median, impaired *in vitro* serum control of KP that was restored by adding healthy serum. $Cfb^{-/-}$ mice demonstrated increased extrapulmonary dissemination and serum inflammatory markers after intratracheal KP infection compared with wild type.

Conclusions: Elevated AP function is associated with improved survival during critical illness, possibly because of enhanced immune capacity.

Keywords: complement; serum; host defense; pneumonia; critical illness

At a Glance Commentary

Scientific Knowledge on the Subject: The complement system is an evolutionarily ancient component of host defense that can drive dysregulated inflammation. The alternative complement pathway, in particular, is important for its ability to amplify complement activity and for its role in the hyperacute response to infection, including host defense in the lung. Little is known about the function of the alternative complement pathway during critical illness.

What This Study Adds to the Field: Critically ill patients exhibit a wide distribution of serum alternative complement pathway function, and those with elevated function demonstrate lower 30-day mortality and improved 1-year survival. Patients with elevated alternative pathway function displayed higher systemic levels of factor B, properdin, factor H, and a "hypoinflammatory" subphenotype, demonstrating fewer bloodstream infections and improved serum control of a multidrug-resistant gram-negative pathogen in vitro. Genetic deletion of factor B or complement component C3, which is crucial for alternative pathway amplification, increased extrapulmonary dissemination of bacteria, and enhanced inflammation in a mouse pneumonia model. These findings suggest that increased fluid-phase alternative complement pathway function is a protective mechanism during the biological stress of critical illness.

The complement system comprises an intricately regulated set of more than 40 proteins that are ancient components of the vertebrate immune system (1). Complement can act both in the "fluid-phase" of blood as well as at a cellular-molecular level in tissues throughout the body (1, 2). There are three well-characterized pathways of complement proteolysis and activation: the alternative, classical, and lectin pathways. The classical pathway is initiated primarily by antigen immunoglobulin complexes. In contrast, the alternative pathway is spontaneously activated but tightly controlled by regulatory proteins and protective host-cell surface inhibitory proteins. Therefore, the function of the complement system may be determined by a delicate balance between positive and negative regulation.

The alternative complement pathway is critically important for its ability to amplify complement activity (3, 4) and for its role in the hyperacute response to infection (5), including host defense in the lung (6, 7). Biological stressors, including infection (8–11), ischemia (12), trauma (13, 14), burns (15), and surgery (16, 17), have all been noted to activate the human complement system. These biological stressors can perturb the complement functional balance, leading to abnormal complement pathway function (8, 18–20) that may have clinical significance (21). Alternative complement pathway function, or AH50, can be quantified by measuring the hemolysis of rabbit erythrocytes after incubation with serum (22). Despite its role in immune function, overexuberant activation of the complement system is thought to contribute to the pathogenesis of sepsis and other critical illnesses (23, 24). We hypothesized that critical illness is associated with perturbations in alternative complement function and sought to characterize the clinical consequences of alternative pathway function. Some of the results of these studies have been previously reported in the form of an abstract (25, 26).

Methods

Subjects

We conducted a retrospective analysis of prospectively collected serum samples from 321 consecutively enrolled patients at the time of enrollment in the University of Pittsburgh Acute Lung Injury Registry and Biospecimen Repository (Institutional Review Board number PRO10110387) from October 2011 to December 2017 (see Methods and Figure E1 in the online supplement).

Microscaled Complement Functional Assays

We made minor modifications to manufacturer (Complement Tech) protocols to perform assays with a limited volume of patient serum and a standard, clear plastic, 96-well plate. Pooled reference serum from healthy volunteers was used to calibrate normal values of complement function for each assay (see Methods in the online supplement). Specific factor-depleted sera (Complement Tech) were used in validation and serum-mixing bacterial killing assays. The microscaled classical (CH50; see Figure E2A) and alternative (AH50; see Figure E2B) pathway function of serum from healthy volunteers were within the normal ranges provided by manufacturer.

Inflammatory Subphenotype Classification

Patients were classified into two distinct inflammatory subphenotypes, as previously described (27). Briefly, "hypoinflammatory"

and "hyperinflammatory" phenotypes were identified by a previously validated three-variable model using IL-8, bicarbonate, and tumor necrosis factor receptor-1 values (28), which demonstrated excellent agreement with *de novo* latent class analysis models incorporating both clinical and biomarker variables in this cohort (27).

Alternative Complement Pathway Protein Analysis and Proximal Regulator Transcriptomics

Serum factor B, properdin, and factor H levels were determined by sandwich ELISA (*see* Methods in the online supplement). Publicly available gene expression data extracted from the EARLI (Early Assessment of Renal and Lung Injury) (29) and MARS (Molecular Diagnosis and Risk Stratification of Sepsis) (30) cohorts were examined in survivors and nonsurvivors (*see* Methods in the online supplement).

Serum Bacterial Growth Assay

Carbapenemase-producing *Klebsiella pneumoniae* (KPC5) clinical isolate from the bronchial washings of a critically ill patient is resistant to meropenem and colistin. The ability of various sera to control KPC5 growth was tested, as previously described (*see* Methods in the online supplement) (31).

Mouse Model

Wild-type (WT) C57BL/6J mice and mice genetically deficient for $C3^{-/-}$ (complement component $C3$; B6.129S4-C3tm1Crr/J, stock number 029661) were purchased from Jackson Laboratory and maintained using a protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee. In separate experiments, WT C57BL/6J and CFB (complement factor B) ($Cfb^{-/-}$)-deficient mice (32, 33) were bred and maintained under pathogen-free conditions at Washington University School of Medicine in St. Louis, MO, in accordance with institutional animal care guidelines. Eight-week to 12-week old, sex-matched mice were used in all experiments. Mice were intratracheally inoculated with a reference hypervirulent *K. pneumoniae* strain (43816; American Type Culture Collection) or KPC5. Necropsy with lung and spleen colony-forming unit counts; and, in select experiments, serum, BAL fluid, and lung tissue for cytokine analysis were collected at 24 hours postinfection, as previously described (31, 34, 35). Serum cytokine measurement was performed by multiplex assay, as previously described (*see* Methods in the online supplement) (36).

Statistical Analysis and Rigor

All assays were performed using deidentified serum specimens by researchers blinded to the clinical characteristics and outcomes of the associated registry patients. Distribution of continuous variables was assessed for normality and transformed if required. Student's *t* tests and chi-squared tests were used to compare continuous and categorical variables between two groups of patients with high and low alternative pathway function defined by median AH50. Associations between complement activity and ICU and 30-day mortality were assessed using logistic regression. Probability of ICU death was calculated from postestimation after fitting a logistic model to confounders (*see* Methods in the online supplement), which was applied as a fractional polynomial function to measure association with ICU mortality. The associations with 1-year survival were assessed with Cox regression analysis (using time to death or last observation). Kruskal-Wallis test with Dunn's test for multiple comparisons was applied to compare serum levels of factor B, properdin, and factor H in a subset of patients, as well as bacterial growth in healthy and factor-depleted sera. Cluster analysis of patients from external cohorts was conducted using hierarchical clustering with complete linkage (stop parameters by Duda-Hart indices) (37). The association between complement activity and bloodstream infection was tested using a multilevel mixed effect logistic regression model. Serum KPC5 killing was tested using a multilevel mixed effect linear regression model. We applied a mean-centering method to remove batch effect on complement activity (38). All analyses were performed after adjusting for confounders, which were selected among those variables considered plausible confounders.

Table 1. Clinical Characteristics of 321 Critically Ill Patients Grouped by Median Alternative Complement Pathway Function

	Total (*N* = 321)	AH50 < Median (*n* = 160)	AH50 ⩾ Median (*n* = 161)	*P* Value
Age, yr, median (IQR)	57 (45–67)	56 (45–67)	58 (45–67)	0.56
Sex, F, *n* (%)	142 (44.2)	69 (43.1)	73 (45.3)	0.69
Race, *n* (%)				0.41
White	297 (92.5)	150 (93.8)	147 (91.3)	
Black	24 (7.5)	10 (6.3)	14 (8.7)	
Admission source OSH, *n* (%)	205 (63.9)	107 (66.9)	98 (60.9)	0.26
ARDS, *n* (%)	107 (33.3)	53 (33.1)	54 (33.5)	0.94
Suspected sepsis, *n* (%)	202 (62.9)	99 (61.9)	103 (64.0)	0.70
Modified SOFA*, median (IQR)	7 (5–9)	8 (6–10)	6 (4–8)	<0.001
Chronic liver disease, *n* (%)	33 (10.3)	28 (17.5)	5 (3.1)	<0.001
Immune suppression, *n* (%)	59 (18.4)	29 (18.1)	30 (18.6)	0.91
Active neoplasm, *n* (%)	19 (5.9)	5 (3.1)	14 (8.7)	0.034
ICU mortality, *n* (%)	84 (26.2)	50 (31.3)	34 (21.2)	0.050
30-d mortality, *n* (%)	93 (29.0)	57 (35.6)	36 (22.4)	0.023

Definition of abbreviations: AH50 = alternative complement pathway function; ARDS = acute respiratory distress syndrome; IQR = interquartile range; OSH = outside hospital; SOFA = sequential organ failure assessment score.
Odds ratio for AH50 ⩾ median is 0.58 (95% confidence interval, 0.34–1.00; *P* = 0.050) for ICU mortality and 0.53 (95% confidence interval, 0.31–0.91; *P* = 0.023) for 30-day mortality. Odds ratios are adjusted for AH50 batch effect, presence of active neoplasm, sex, SOFA, and patient age.
*Modified SOFA score calculated with neurologic score equal to 0; therefore, maximum score is 20.

Statistical models were checked for assumptions, including highly influential observations and proportionality of hazards. Statistical analysis was performed in Stata Version 15.1 (StataCorp).

Results

Clinical Characteristics for 321 Critically Ill Patients

Although others have shown perturbations of complement function in small series of patients with bacteremia (19, 39, 40), we sought to determine whether complement pathway function was associated with mortality in a large, heterogenous cohort of critically ill patients with acute respiratory failure. Table 1 displays clinical characteristics of the 321 consecutively enrolled research registry patients (median age, 57 yr; 44% female). Samples were collected within a mean of 2.1 days (SD, 2.2 d) after ICU admission. Consistent with the underlying demographics of the surrounding region, patients were primarily white (93%) and the majority were transferred from local outside hospitals (64%). The vast majority of patients (96%) required mechanical ventilation during their ICU course. Most patients were either at-risk ($n = 125$; 39%) or met diagnostic criteria for acute respiratory distress syndrome (ARDS) ($n = 107$; 33%). The remaining patients ($n = 89$; 28%) were mechanically ventilated for nonpulmonary critical illnesses such as heart failure or encephalopathy. Sepsis was confirmed or probable in 63% ($n = 202$) of patients and
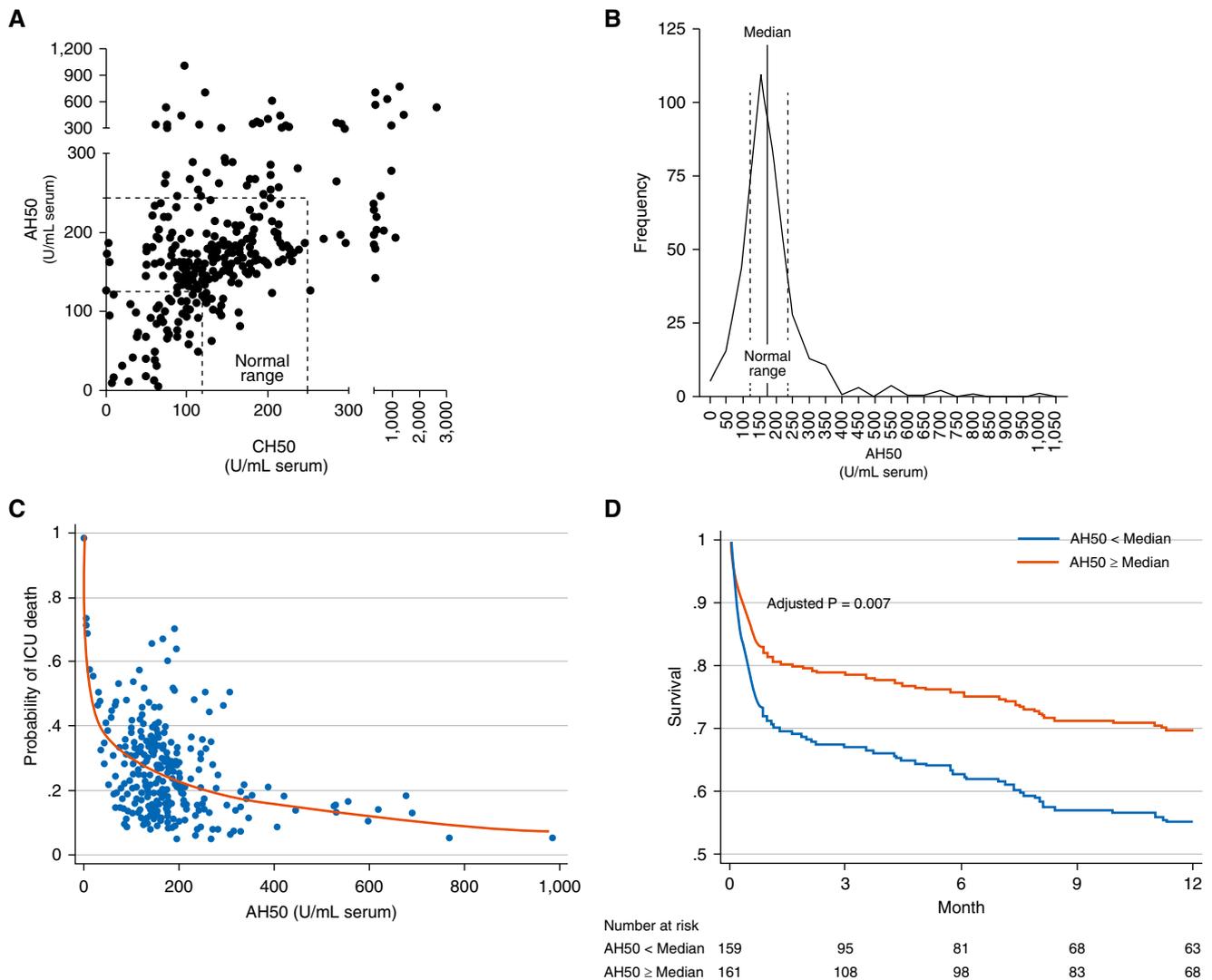
Figure 1. Increased alternative complement pathway activity is associated with decreased 30-day mortality and increased 1-year survival. (*A*) Scatter plot of classical pathway function (CH50) (U/ml serum; median, 130; interquartile range, 90–179) and alternative pathway function (AH50) (U/ml serum; median, 169; interquartile range, 136–204) values and (*B*) frequency distribution of AH50 values (grouped by increments of 50 U/ml serum; median value displayed) of serum prospectively collected from 321 consecutively enrolled critically ill patients at the time of enrollment. Normal value ranges are supplied by the manufacturer. (*C*) Probability of ICU mortality as a function of batch-adjusted AH50 ($P = 0.015$). (*D*) Patient survival from index ICU admission date by AH50 grouped by relationship to median (hazard ratio, 0.59 [95% confidence interval, 0.41–0.87]). Survival estimates are adjusted for AH50 batch effect, patient age, sex, sequential organ failure assessment score, chronic liver disease, and presence of active neoplasm. For AH50 less than median ($n = 159$), a single patient died within 24 hours of ICU admission and was therefore removed from this analysis rather than allocating an arbitrary duration of follow-up.

the median modified sequential organ failure assessment (SOFA) score was 7 (interquartile range, 5–9). We noted chronic liver disease (n = 33; 10%), immune suppression (n = 59; 18%), and active neoplasm (n = 19; 6%) in some patients. Ninety-three of 321 (29%) patients died within 30 days of their admission to the ICU.

Values of CH50 and AH50 from serum collected at the time of enrollment were significantly correlated (Figure 1A; Spearman r = 0.50; P < 0.0001). Figure 1B displays the frequency distribution of AH50 values (range 6–1,008 U/ml serum). Given the wide distribution of complement function values, we examined whether there was an association between clinical outcomes and complement function in our registry patients.

Complement Pathway Function Is Associated with Mortality
After adjusting for age, sex, SOFA, presence of active neoplasm, and batch effect, we found significantly decreased ICU (odds ratio [OR], 0.58 [95% confidence interval (CI), 0.34–1.00]; P = 0.050) and 30-day mortality (OR, 0.53 [95% CI, 0.31–0.91]; P = 0.023) in patients with AH50 greater than or equal to median (see Table 1). There were no significant differences in ARDS diagnosis, or confirmed or suspected sepsis, between groups, although modified SOFA scores were higher in patients with AH50 less than median. Increased numbers of patients diagnosed with chronic liver disease showed AH50 less than median (P < 0.001). However, the increased mortality observed in patients with lower alternative pathway function was not impacted by the presence of chronic liver disease in multivariate analysis (OR for 30-d mortality adjusted for chronic liver disease = 0.52 [95% CI, 0.30–0.90]; P = 0.020). Furthermore, when patients with history of chronic liver disease were removed from the analysis (n = 33 patients), the association between reduced 30-day mortality and higher AH50 was preserved in the remaining 288 patients (OR, 0.53 [95% CI, 0.30–0.93]; P = 0.027).

In contrast to AH50, we did not find a significant relationship between CH50 greater than or equal to median and ICU mortality (adjusted OR, 1.22 [95% CI, 0.72–2.09]; P = 0.46) nor 30-day mortality (adjusted OR, 1.02 [95% CI, 0.60–1.73]; P = 0.96) after adjustment for age, sex,

presence of active neoplasm, SOFA, and batch effect. AH50 level as a continuous variable was associated with ICU mortality with decreasing probability of death in patients with higher complement pathway function (see Figure 1C; P = 0.015), whereas CH50 level was not predictive of ICU mortality (see Figure E3A; P = 0.31).

Others have shown associations between alternative pathway regulatory protein levels and long-term outcomes in patients with systolic heart failure (41). Therefore, we investigated differences in 1-year survival based on alternative pathway function. We found that AH50 greater than or equal to median was associated with increased 1-year survival (see Figure 1D; hazard ratio [HR], 0.59 [95% CI, 0.41–0.87]; adjusted P = 0.007) after multivariate adjustment, including the presence of chronic liver disease. Notably, CH50 values were not significantly associated with 1-year survival (see Figure E3B; HR, 1.00 [95% CI, 0.69–1.44]; adjusted P > 0.9). Limiting analysis to patients with sepsis (n = 202) provided a stronger survival association in patients with AH50 greater than or equal to median (HR, 0.51 [95% CI, 0.32–0.79]; adjusted P = 0.003).

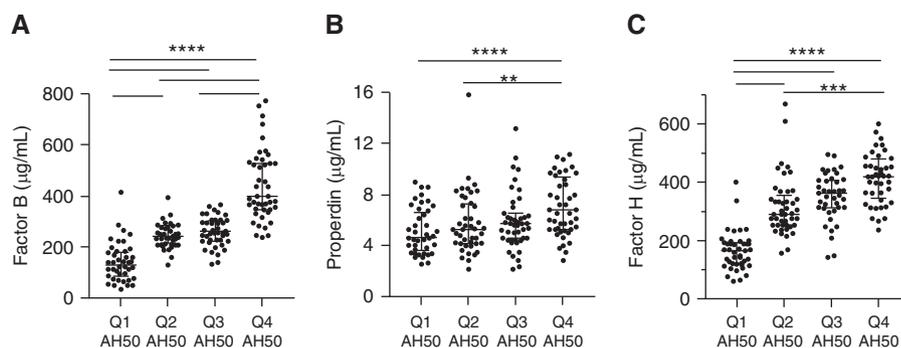### Increased Alternative Complement Pathway Activity Is Associated with a Hypoinflammatory Subphenotype
Others have shown clinical subsets of ARDS patients characterized
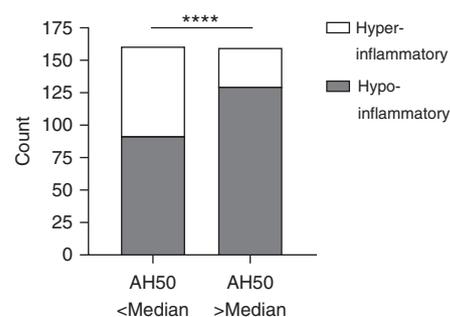
Figure 2. Increased alternative complement pathway activity is associated with a hypoinflammatory phenotype. Alternative pathway function (AH50) greater than or equal to median is associated with a hypoinflammatory phenotype (odds ratio, 0.30 [95% confidence interval, 0.18–0.49]) as classified by a three-variable model consisting of bicarbonate, IL-8, and tumor necrosis factor receptor-1. Statistical analysis was by chi-square test. ****P < 0.0001.

by hyperinflammatory versus hypoinflammatory subphenotypes, with implications for mortality during critical illness (28, 42). Notably, established ARDS subphenotypes may offer prognostic information, not only in patients with ARDS but also in mechanically ventilated patients at risk for ARDS (27).

Therefore, we classified the inflammatory subphenotypes of 321 patients in our cohort using a previously validated model (28). Patients with elevated alternative complement activity were less likely to exhibit a

Figure 3. Increased alternative complement pathway function (AH50) is associated with increased serum levels of both positive and negative regulatory fluid-phase proteins. Sera from 181 patients of 321 patients (n = 44, [quartile 1] Q1; n = 45, Q2; n = 45, Q3; and n = 47, Q4) were characterized for (A) factor B (μg/ml; Q1 median, interquartile range: 133, 92–176; Q4: 394, 347–526; P < 0.0001 for Q4 compared with all other quartiles), (B) properdin (μg/ml; Q1: 4.7, 3.7–6.5; Q4: 6.8, 5.4–9.3; P < 0.0001 compared with Q1), and (C) factor H (μg/ml; Q1: 166, 122–189; Q4: 419, 354–471; P < 0.0001 compared with Q1 and Q2). Statistical analysis was by Kruskal-Wallis test with Dunn's *post hoc* test for multiple comparisons; horizontal lines represent *post hoc* statistical relationships. **P < 0.01, ***P < 0.001, and ****P < 0.0001.

hyperinflammatory subphenotype (Figure 2; OR, 0.30 [95% CI, 0.18–0.49]; $P < 0.0001$). We therefore sought to better understand the regulatory mechanisms that determine alternative pathway function in our cohort.

Increased Alternative Complement Pathway Function Is Associated with Increased Serum Levels of Both Positive and Negative Regulatory Fluid-Phase Proteins

To investigate potential regulatory mechanisms that influence alternative pathway function, we examined serum levels of three major alternative pathway regulatory proteins within a stratified sample of patients from each quartile ($N = 181$). We found patients with the highest AH50 values had significantly higher levels of factor B (Figure 3A; Pearson correlation coefficient = 0.74; $P < 0.001$) and the positive regulator, properdin (see Figure 3B; Pearson correlation coefficient = 0.38; $P < 0.001$). Similarly, patients with the highest AH50 values had significantly higher levels of the negative regulator factor H (see Figure 3C; Pearson correlation coefficient = 0.58; $P < 0.001$). We note a trend toward improved survival in patients with factor B and factor H greater than the median that did not remain significant after multivariate adjustment (see Figure E4). Given the importance of alternative pathway regulatory factors in determining alternative pathway function, we next examined the transcriptional profile of alternative pathway regulatory factors in two external cohorts.

Increased Transcription of Fluid-Phase Alternative Pathway Components Is Associated with Improved Survival in Two External Critically Ill Cohorts

Both the EARLI and MARS cohorts report whole-blood transcriptional data of alternative pathway proximal *CFD* (complement factor D), *CFB*, and *CFP* (complement factor properdin) genes in critically ill patients with acute respiratory failure. We found increased relative expression of *CFD*, *CFB* (three separate transcripts), and *CFP* (Figure 4A) in EARLI patients who survived to 60 days. Similarly, we found increased relative expression of *CFD* and *CFP* in MARS patients who
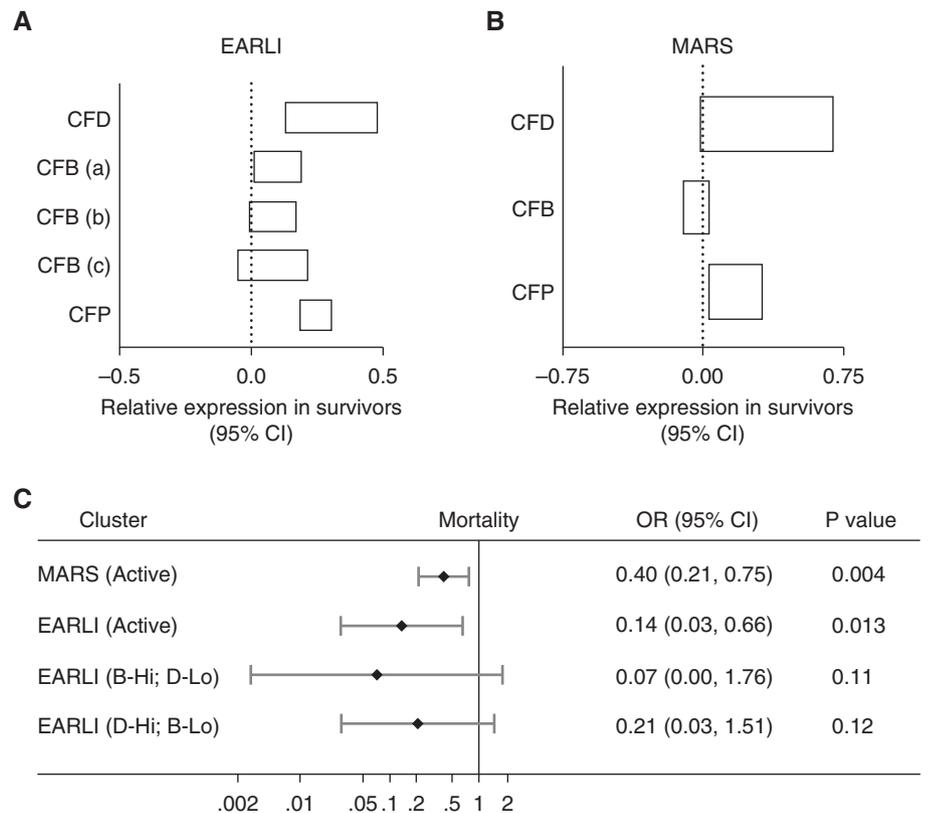
Figure 4. Increased transcription of fluid-phase alternative pathway components is associated with improved survival in two external critically ill cohorts. Whole-blood transcription levels of *CFD* (complement factor D), *CFB* (complement factor B), and *CFP* (complement factor properdin) genes were increased in survivors of critical illness in the (*A*) EARLI (Early Assessment of Renal and Lung Injury) ($n = 62$; 48 survivors at 60 d) and (*B*) MARS (Molecular Diagnosis and Risk Stratification of Sepsis) ($n = 479$; 365 survivors at 28 d) cohorts as measured by Affymetrix gene array and published on the National Center for Biotechnology Information Gene Expression Omnibus. The 95% CI of relative gene expression as measured by *t* test is displayed. There are three separate *CFB* gene transcripts listed as *CFB* (a), *CFB* (b), and *CFB* (c) included in the EARLI data. (*C*) Cluster analysis using hierarchical clustering with complete linkage (stop parameters by Duda-Hart indices) revealed two MARS cohort clusters (active marked by increased *CFD* and *CFP* expression) and four EARLI cohort clusters (active marked by increased *CFD*, *CFB*, and *CFP* expression; B-Hi; D-Lo marked by elevated *CFB* and decreased *CFD* expression; and D-Hi; B-Lo marked by elevated *CFD* and decreased *CFB* expression) with decreased risk of mortality compared with the reference cluster in each cohort as measured by logistic regression. CI = confidence interval; OR = odds ratio.

survived to 28 days. Because the AH50 is a total functional characterization of several proteins that contribute to alternative pathway activity, we sought to model alternative pathway activation by analysis of hierarchical clustering of gene expression data. Two clusters were identified in MARS: one cluster marked by elevated *CFD* and *CFP* expression ("Active"; $n = 433$) demonstrated decreased 28-day mortality (OR, 0.40 [95% CI, 0.21–0.75]; $P = 0.004$) compared with the cluster with lower expression. Four clusters were identified in EARLI: one

cluster with elevated expression of *CFD*, *CFB*, and *CFP* ("Active"; $n = 41$) demonstrated decreased mortality (OR, 0.14 [95% CI, 0.03–0.66]; $P = 0.013$) compared with the reference cluster with decreased expression. Two other clusters, one with elevated *CFB* but decreased *CFD* ("B-Hi; D-Lo", $n = 4$) and the other with elevated *CFD* but decreased *CFB* ("D-Hi; B-Lo", $n = 9$) demonstrated a trend toward decreased mortality (OR, 0.07 [95% CI, 0.00–1.76] and OR, 0.21 [95% CI, 0.03–1.51], respectively) but did not reach statistical significance.

Table 2. Increased Alternative Complement Pathway Function Is Associated with Decreased Positive Blood Culture Burden

Pathogen	Positive Blood Culture [n (% Patients with Blood Culture)]	AH50 ⩾ Median [OR (95% CI)]	P Value	Adjusted* [OR (95% CI)]	P Value
Gram-negative bacteria[†]	20 (7.1)	0.67 (0.42–1.08)	0.10	0.72 (0.44–1.19)[‡§]	0.20
Gram-positive bacteria[‖]	31 (11.0)	0.63 (0.34–1.15)	0.13	0.73 (0.42–1.27)	0.27
Yeast[¶]	6 (2.1)	0.53 (0.21–1.35)	0.19	0.62 (0.23–1.70)[‡§]	0.35
Any bacteria	46 (16.3)	0.68 (0.46–1.02)	0.063	0.71 (0.48–1.05)	0.082
Any bacteria or yeast	48** (17.0)	0.65 (0.44–0.97)	0.034	0.67 (0.45–0.98)	0.040

Definition of abbreviations: AH50 = alternative complement pathway function; CI = confidence interval; OR = odds ratio.
Of 321 cohort patients, 283 had one or more blood cultures drawn from 24 hours before and 30 days after ICU admission. Multiple positive blood cultures collected on the same day were combined into a single result per organism.
*Values were adjusted for age, sex, sequential organ failure assessment score, duration of ICU admission, total number of blood cultures drawn, the presence of chronic liver disease, and suspected sepsis.
[†]Gram-negative bacteria include *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *K. variola*, *Pseudomonas aeruginosa*, and *Serratia marcescens*.
[‡]Presence of chronic liver disease is not included in adjustment because the OR could not be calculated because no patients with chronic liver disease had gram-negative bacteria or yeast bloodstream infections.
[§]Suspected sepsis is not included in adjustment because all patients with positive cultures were considered confirmed or probable cases of sepsis.
[‖]Gram-positive bacteria include *Enterococcus faecalis*, *E. faecium*, *Staphylococcus aureus* (both methicillin-susceptible and methicillin-resistant), *Streptococcus gallolyticus*, and *Streptococcus pneumoniae*.
[¶]Yeast include *Candida albicans*, *C. glabrata*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, and *C. tropicalis*.
**There were 48 patients with at least one positive culture, 9 of whom had more than one, for a total of 57 positive cultures.

Increased Alternative Complement Pathway Function Is Associated with Fewer Bloodstream Infections and Improved Serum Killing of a Nosocomial Lung Pathogen

To investigate whether complement activity influences the risk of infection during critical illness, we investigated the association between complement pathway function and the presence of documented bloodstream infection. The majority of patients (283 of 321; 88%) had one or more blood cultures drawn up to 24 hours before, or within 30 days after, ICU admission.

Of the 48 (17%) patients with a blood culture positive for a pathogenic bacteria or yeast (Table 2), AH50 greater than or equal to median was associated with decreased risk of bloodstream infection (adjusted OR, 0.67 [95% CI, 0.45–0.98]; $P = 0.040$). We sought to further explore the role of
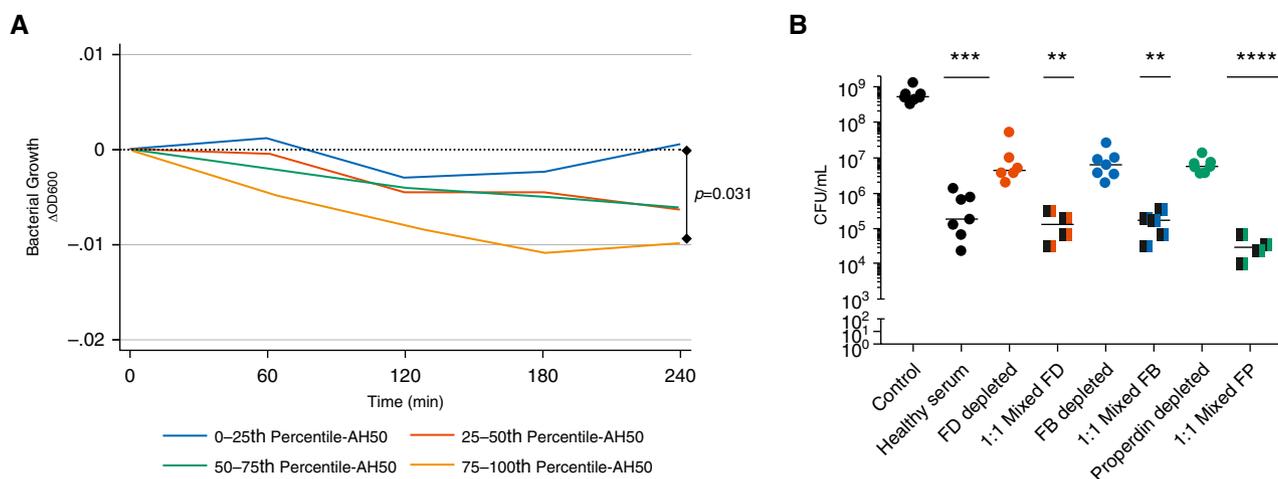
Figure 5. The alternative complement pathway contributes to *in vitro* serum control of an extensively drug-resistant *Klebsiella pneumoniae* lung pathogen. (*A*) Bacterial growth of the carbapenemase-producing *K. pneumoniae* clinical isolate KPC5 as measured by change in optical density 600 nm when grown in tryptic soy broth with 85% vol/vol serum from critically ill patients ($n = 321$). Median optical density 600 nm by quartile of AH50 is displayed. The association between complement activity and KPC5 growth was assessed by a multilevel mixed-effects linear regression model. (*B*) In separate experiments, KPC5 was grown for 4 hours in 5% vol/vol tryptic soy broth, serially diluted, plated, and counted (cfu/ml). Groups include KPC5 grown in 85% vol/vol solution of sterile phosphate buffered saline (control), healthy serum, and sera depleted of factor D (FD), factor B (FB), or properdin (FP) ($n = 7$ trials). In select experiments, healthy serum was also mixed 1:1 with sera depleted of FD ($n = 4$ trials), FB ($n = 5$ trials), and FP ($n = 4$ trials). Each point represents a single trial. Statistical analysis by Kruskal-Wallis test with Dunn's *post hoc* significance compared with control is displayed. **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$. AH50 = alternative complement pathway function; ΔOD600 = change in optical density at 600 nm.
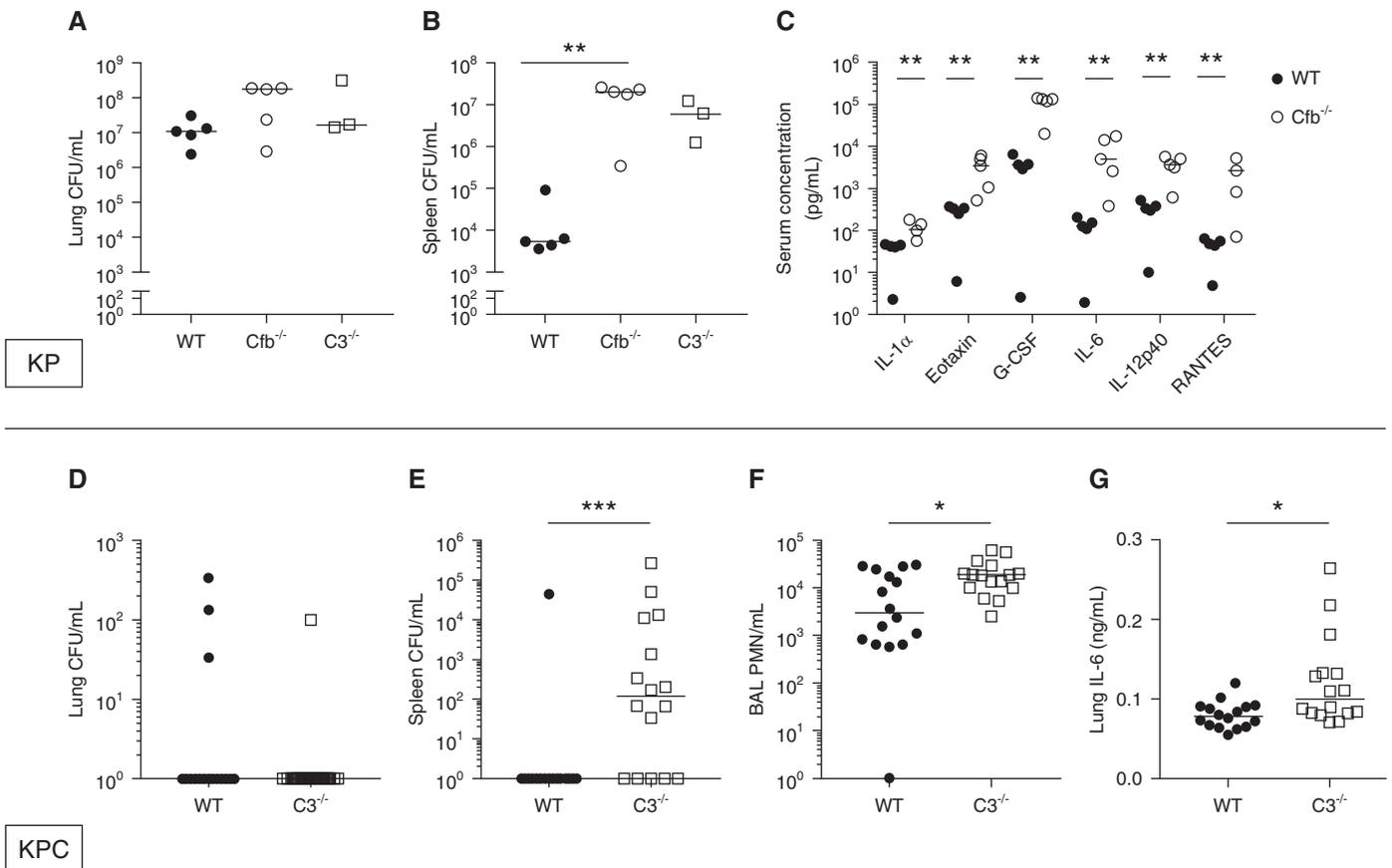
Figure 6. $Cfb^{-/-}$ and $C3^{-/-}$ mice demonstrate increased splenic dissemination of *Klebsiella pneumoniae* (KP) after acute intrapulmonary infection with both a hypervirulent and a nosocomial KPC strain. Wild-type (WT) ($n = 5$), $Cfb^{-/-}$ ($n = 5$), and $C3^{-/-}$ ($n = 3$) mice were intratracheally inoculated with 10^6 cfu of KP2 (American Type Culture Collection 43816), a hypervirulent reference KP strain. (*A*) Lung bacterial cfu/ml, (*B*) spleen cfu/ml, and (*C*) serum concentration (pg/ml) of select cytokines by multiplex obtained at necropsy 24 hours postinfection. In separate experiments, WT and $C3^{-/-}$ mice ($n = 16$ mice each group from two separate experiments) were intratracheally inoculated with 10^3 cfu of KPC5, a clinical isolate with muted virulence that is resistant to all tested antibiotics, and necropsy was performed at 24 hours. (*D*) Lung bacterial cfu/ml, (*E*) spleen bacterial cfu/ml, (*F*) BAL polymorphonucleated cells (PMN)/ml, and (*F*) lung IL-6 (ng/ml). Each point represents an individual mouse. (*A* and *B*) Statistical analysis by Kruskal-Wallis test with Dunn's *post hoc* statistical significance compared with WT group displayed. (*C–G*) Statistical analysis by Mann-Whitney test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. KPC = carbapenemase-producing *Klebsiella pneumoniae*; RANTES = regulated upon activation, normal T cell expressed and secreted.

complement function in host defense using a serum sensitivity assay. We used an extensively drug-resistant (XDR) nosocomial lung pathogen, *K. pneumoniae* strain KPC5, that is resistant to all antibiotics including meropenem and colistin but is sensitive to killing by serum from healthy volunteers (31). We found that serum from patients with elevated AH50 (quartile 4) demonstrated decreased growth of KPC5 *in vitro* compared with serum from patients with low AH50 (Figure 5A). Conversely, serum depleted of alternative complement pathway components factor D, factor B, and properdin demonstrated impaired ability to kill KPC5 that was restored by

mixing equal parts serum from healthy volunteers (*see* Figure 5B).

$Cfb^{-/-}$ and $C3^{-/-}$ Mice Demonstrate Increased Splenic Dissemination of *K. pneumoniae* after Acute Intrapulmonary Infection

To further explore the hypothesis that alternative complement pathway function supports host defense during infection, we used an acute intrapulmonary infection model with *K. pneumoniae* strains in mice genetically deficient for factor B ($Cfb^{-/-}$), which effectively knocks out the alternative complement pathway, and C3 ($C3^{-/-}$), which is crucial for alternative pathway amplification of all complement activity. We found similar lung bacterial burden

(Figure 6A) but significantly increased splenic dissemination of a hypervirulent KP strain (ATCC 43816) 24 hours after intratracheal inoculation in $Cfb^{-/-}$ mice compared with WT (*see* Figure 6B), which was phenocopied in $C3^{-/-}$ mice. Serum cytokines revealed significantly elevated IL-1α, eotaxin, granulocyte colony-stimulating factor, IL-6, IL-12p40, and RANTES (regulated upon activation, normal T cell expressed and secreted) (*see* Figures 6C and E5) in $Cfb^{-/-}$ mice compared with WT. In separate experiments, we found minimal lung bacterial counts of the relatively avirulent XDR lung pathogen KPC5 in WT and $C3^{-/-}$ mice 24 hours postinfection (*see* Figure 6D). However, $C3^{-/-}$ mice

demonstrated significantly increased splenic dissemination of KPC5 (*see* Figure 6E). Despite similar lung bacterial burden, $C3^{-/-}$ mice demonstrated higher BAL polymorphonuclear cell counts (*see* Figure 6F) and increased lung concentration of the cytokine IL-6 (*see* Figure 6G). There was no significant difference in BAL fluid protein or IgM levels between groups (*see* Figure E6).

Discussion

We examined the function of the classical and alternative complement pathways in 321 critically ill patients using a serum-sparing microscaled assay, which represents the largest data set characterizing complement function in a critically ill cohort to date. We found a wide distribution of complement function in our cohort and a novel association between increased alternative pathway function at ICU admission and reduced 30-day mortality. We further demonstrated that higher alternative pathway function was associated with increased survival one year after ICU admission. Patients with elevated alternative pathway function displayed a hypoinflammatory subphenotype, and higher levels of factor B and the positive regulatory protein, properdin, as well as the negative regulator, factor H. We found increased transcription of genes for proximal alternative pathway components factor D, factor B, and properdin in survivors of critical illness in publicly available data from two external cohorts. Within our cohort, there were fewer bloodstream infections in patients with AH50 greater than or equal to median, and serum from patients with AH50 greater than or equal to median showed enhanced control of XDR pathogen growth *in vitro*. Conversely, serum depleted of alternative pathway factors showed impaired control of the same XDR lung pathogen *in vitro* that corrected with the addition of healthy serum. Finally, we demonstrated that genetic deletion of factor B or *C3*, which is crucial to alternative pathway amplification, impairs host defense against extrapulmonary dissemination during acute intrapulmonary infection models in mice. Taken together, these findings suggest that increased fluid-phase alternative complement pathway function is a protective mechanism during the biological stress of critical illness.

Although the complement system has been previously examined in preclinical and clinical investigations of critical illness and respiratory failure (8–11, 18), few have addressed a specific role for alternative complement pathway function in clinical outcomes. Although others have studied complement activation in ARDS (43–46), we did not observe any difference in the frequency of ARDS in patients with AH50 greater than or equal to median and AH50 less than median in our cohort. Rather than investigating complement activation, we used the AH50 functional assay, which provides a global assessment of both positive and negative regulatory factors that determine total alternative complement pathway function. We propose two potential mechanisms for the observed benefit of increased alternative complement pathway function. First, elevated alternative complement pathway function could provide improved host defense, which may be crucial to protection during critical illness. Several preclinical models and clinical investigations have demonstrated that deficiencies in alternative complement function can impair host defense against pathogens (6, 7, 47–50). Furthermore, prior studies in humans during critical illness have identified upregulation of alternative complement and innate immune pathways in the glycoproteomic profile of sepsis survivors (21) and an association between genetic disposition toward alternative complement activation and decreased frequency of sepsis in critically ill children (51). Notably, the impaired ability of serum with low AH50 to control an XDR *K. pneumoniae* lung isolate invites the possibility that diminished alternative complement function during critical illness may increase the risk of some nosocomial infections. It is also possible that intact complement function may contribute to improved outcomes beyond the direct antimicrobial role of complement by supporting immune cell regulation such as cellular proliferation, metabolism, and survival (52, 53). For example, one intriguing concept is that, although immune cells can directly release complement into tissue microenvironments, circulating fluid-phase complement components may replenish complement activity in tissue microenvironments, contributing to critical local functions, including in the lung (2, 54). Therefore, the alternative complement pathway likely has important roles in host defense during critical illness.

The second mechanism of benefit we propose is that elevated alternative pathway function may imply a host with the ability to both rapidly initiate and deescalate an inflammatory response. In support of this concept, we found that patients with elevated alternative pathway function were more likely to demonstrate a hypoinflammatory subphenotype, which appears consistent with a recent report that elevated *CFD* transcription is associated with a beneficial subphenotype in patients with ARDS (30). Furthermore, we note the unexpected finding that patients with elevated alternative pathway function had elevated levels of a crucial negative regulator of the alternative pathway, factor H (55). Circulating levels of factor H have been reported to be stable between illness and recovery (11), and others have reported that factor H is not an acute phase reactant (56). Interestingly, we found factor H levels that are similar to prior reports in both critically ill patients (11) and stable outpatients (57–61). Therefore, it is possible that baseline factor H levels may be a major determinant of complement function during critical illness. Taken together, we propose that the ability to simultaneously initiate a robust host defense and deescalate an overly exuberant inflammatory response may have important implications for clinical outcomes (62–64).

Our study has several strengths, including characterization of total alternative complement pathway function in a heterogenous cohort of critically ill patients, evaluation of complement function and mortality, and translational models demonstrating potential mechanisms of protection provided by alternative complement function. However, one limitation is that findings of total alternative complement function are from a single academic medical center cohort. Notably, the data are supported by findings from two external cohorts that demonstrated increased transcription of proximal alternative pathway regulators in survivors of critical illness.

Conclusions
We have shown that increased alternative complement pathway function is associated with improved survival during critical

illness, possibly due to improved host immune capacity. Further work remains to better define the biologic role of alternative complement pathway function during critical illness and to consider whether those pathways can be modulated in pursuit of improved patient outcomes (65). ■

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