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AUGMENTED PASSIVE IMMUNOTHERAPY WITH P4 PEPTIDE IMPROVES PHAGOCYTE ACTIVITY IN SEVERE SEPSIS

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ABSTRACT—**Introduction:** Antimicrobial resistance threatens to undermine treatment of severe infection; new therapeutic strategies are urgently needed. Preclinical work shows that augmented passive immunotherapy with P4 peptide increases phagocytic activity and shows promise as a novel therapeutic strategy. Our aim was to determine *ex vivo* P4 activity in a target population of patients admitted to critical care with severe infection. **Methods:** We prospectively recruited UK critical care unit patients with severe sepsis and observed clinical course ($\geq$3 months postdischarge). Blood samples were taken in early ($\leq$48 h postdiagnosis, n = 54), latent (7 days postdiagnosis, n = 39), and convalescent (3–6 months postdiagnosis, n = 18) phases of disease. The primary outcome measure was killing of opsonized *Streptococcus pneumoniae* by neutrophils with and without P4 peptide stimulation. We also used a flow cytometric whole blood phagocytosis assay to determine phagocyte association and oxidation of intraphagosomal reporter beads. **Results:** P4 peptide increased neutrophil killing of opsonized pneumococci by 8.6% (confidence interval 6.35–10.76, $P < 0.001$) in all phases of sepsis, independent of infection source and microbiological status. This represented a 54.9% increase in bacterial killing compared with unstimulated neutrophils (15.6%) in early phase samples. Similarly, P4 peptide treatment significantly increased neutrophil and monocyte intraphagosomal reporter bead association and oxidation, independent of infection source. **Conclusions:** We have extended preclinical work to demonstrate that P4 peptide significantly increases phagocytosis and bacterial killing in samples from a target patient population with severe sepsis. This study supports the rationale for augmented passive immunotherapy as a therapeutic strategy in severe sepsis.

KEYWORDS—Bacterial infection, innate immunity, neutrophil biology

INTRODUCTION

Severe sepsis is a worldwide public health concern despite recent advances and standardization in treatment (1–3). The current evidence base for effective therapies is limited to antibiotics, source control, and organ support (4). In this context, antimicrobial resistance is of immediate concern and has been described as ‘‘a threat to national security’’ by prominent health leaders (5). Augmented passive immunotherapy using a peptide fragment of pneumococcal surface adhesin A (PsaA) to stimulate phagocytic cells is one alternative therapeutic strategy that shows promise (6).

P4 peptide is a 28–amino acid fragment of PsaA, a protein expressed on the surface of *Streptococcus pneumoniae*. Initially investigated as a vaccine candidate, it was discovered that P4 peptide stimulation promotes increased bacterial (including *S pneumoniae*, *Streptococcus pyogenes*, *Neisseria meningitidis*, and *Staphylococcus aureus*) binding by phagocytic cells (7, 8). Following this, multiple *in vivo* investigations have shown that the administration of P4 peptide in combination with immunoglobulin rescues moribund mice from death in streptococcal and staphylococcal infection models (8–14). Furthermore, healthy human alveolar macrophages demonstrate improved

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BM and EM contributed equally to this work.

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bacterial killing when exposed to P4 peptide *ex vivo* (15). However, patients with severe infection develop marked immune dysregulation, negatively impacting on innate and adaptive immune function (16). Thus, it is unclear if the effects of P4 peptide observed *in vitro* and *in vivo* will also apply to our target population—patients with severe sepsis.

The aim of our study was to test the effect of P4 peptide on phagocytic cells *ex vivo* from patients admitted to critical care with severe sepsis. Determination of P4 peptide activity in this target population will support the translation of this promising therapeutic strategy to future clinical trials and clinical utility.

PATIENTS AND METHODS

Clinical

We recruited patients admitted to critical care with severe sepsis according to ACCP-SCCM 2001 criteria (17) at Aintree University Hospital and Royal Liverpool University Hospital, UK (February 2014 to June 2015). Patient inclusion criteria were as follows: age $\geq$18, recruitment $\leq$48 h diagnosis, and severe sepsis from respiratory, abdominal, or urogenital source (predominant causes (18)). Patient exclusion criteria were as follows: immunocompromising condition or therapy, pregnancy, responsible clinician deemed inappropriate, coenrolment in another study that could have influenced results, or failure to obtain consent. Blood samples were taken at days 0 and 7, and at 3 to 6 months to determine phagocyte function in the early, latent, and convalescent phases of infection. Previous work has demonstrated suppression of neutrophil response in the latent phase of infection (19). Our aim was to capture this phase to determine peptide activity and follow with convalescent samples after patients had been discharged home. Healthy volunteers were recruited and sampled at matched intervals (Clinical Research Facility, Royal Liverpool University Hospital) to determine assay stability and validate components. Healthy volunteer inclusion criteria were as follows: no current illness, aged >18, and able to travel to the research facility. Healthy volunteer exclusion criteria were the same as for patients. The UK NHS Research Ethics Committee granted approval for this project (13/WA/0353). Written consent was obtained for all participants. A CONSORT diagram that details patient screening and inclusion is provided in Figure S1 (Supplemental Digital Content, http://links.lww.com/SHK/A443).

Laboratory

P4 peptide and M12-1 peptide were synthesized and purified by Centers for Disease Control, Atlanta, Ga, and reconstituted in diethylpyrocarbonate (DEPC) water. The primary outcome measure was neutrophil bacterial killing determined by a standardized opsonophagocytosis assay (with minor modifications (20)). Briefly, neutrophils were isolated from heparinized blood by density centrifugation and incubated (45 min, 37°C, shaking 300 RPM) with live pneumococci (capsulated serotype 2, D39 strain), human intravenous immunoglobulin (IVIG; Gamunex, Grifols Inc, Spain), baby rabbit complement (Mast Group, Bootle, UK), and P4 peptide (1 mg/mL) or control (M12-1 peptide 1 mg/mL and DEPC vehicle). The dose of P4 peptide in this assay was based on previously published work (8). The multiplicity of infection was 100:1 (neutrophil:bacteria). Plates were cooled (4°C) to arrest phagocytic activity and the mixture incubated for 18 to 24 h (blood agar, 37°C, 5% CO_2) before colony-forming unit enumeration. All samples were performed in triplicate. Neutrophil bacterial killing index is defined as follows: 1 – (experimental well divided by non-ploymorphonuclear leukocytes [PMN] control well). Experimental components were neutrophils (PMN), opsonized bacteria, and complement with P4 peptide, M12-1 peptide, or vehicle control. Non-PMN components were opsonized bacteria and complement. See Supplemental Digital Content, http://links.lww.com/SHK/A443, for full details of experimental methods.

Our whole blood phagocytosis assay is built on previous work (21). After venepuncture, citrated blood (4.5 mL, BD) was placed on an automated mixer at room temperature. The intraphagosomal reporter beads constitute immunoglobulin G–coated silica beads with calibrator (Pacific Blue, Invitrogen, USA) and reporter (OxyBURST Green, Invitrogen, USA) fluorochromes. Beads were reconstituted (30×10^6/mL) in Roswell Park Memorial Institute media (Invitrogen, USA) and mixed with experimental solution (4 mg/mL P4 peptide or plain DEPC), 5:1 ratio. This dose was based on a P4 peptide titration experiment (Supplemental Digital Content, http://links.lww.com/SHK/A443). Subsequently, paired 120 μL aliquots (0, 10, 20, 30, 45, and 60 min) were incubated with whole blood (100 μL) at 37°C in the dark. Multiple fluorescence minus one controls were used to set negative and positive boundaries for analysis (Fig. S2, Supplemental Digital Content, http://links.lww.com/SHK/A443). After allotted incubation time, samples were rapidly cooled (4°C) to arrest biological activity, red cells treated with lysis buffer (BioLegend, San Diego, Calif), and samples washed before flow cytometric acquisition. In preliminary work, cell surface markers were used to define monocyte populations (CD14, BD Pharmingen, USA) and estimate neutrophil function (CD66b, Stemcell Technologies, Canada). Compensation beads (BD Biosciences, USA) were used to create compensation matrices. Cell populations of interest were identified by sequential cell gating strategies. Samples were acquired using a BD LSRII flow cytometer equipped with three lasers (405 nm, 488 nm, and 633 nm; Becton Dickinson, USA) using FACS-Diva (version 6.1, BD Biosciences, USA) and FlowJo (version 10.7, Tree Star, USA) flow cytometry software.

Statistics

Data were examined for normality before applying appropriate tests. All patient samples were analyzed pairwise (P4 peptide vs. vehicle or peptide control). Mixed model regression analyses were used to allow the hierarchical nature of patient sampling (one patient repeatedly sampled three times). Three mixed models were used for the main analyses: model one had neutrophil bacterial killing (%) as the dependent variable, phase of infection (early, latent, convalescent) and group (P4 peptide, control) as fixed effects, and patient as random effect. Model two had neutrophil intraphagosomal reporter bead association as dependent variables, phase of infection (early, latent, convalescent), group (P4 peptide, control), time (0, 10, 20, 30, 45, 60 min), and interaction between group and time as fixed effects, and patient as random effect. Model three had intraphagosomal reporter bead oxidation ratio as the dependent variable with fixed and random effects as per model 2. The differences between P4 peptide and control in mean of those outcomes at different time points together with 95% confidence interval (CI) were derived from the mixed models. Mixed model regression assumes that missing values are at random. Mann–Whitney *U* test was used when normality assumption was violated. All data were analyzed using STATA 13.1 (Statacorp, USA).

RESULTS

Patient recruitment—clinical details and microbiology

Fifty-four patients were recruited to this study; 30 (55.6%) were men and median age was 63.5 (interquartile range [IQR] 53–75). Fifty-two patients were of white ethnic origin, one Asian, and one black. There were 17 smokers and median body mass index was 27.3 (IQR 24.0–31.3). Premorbid status was quantified using Charlson Index score (22) (median 2, IQR 1–3). The most common premorbid conditions were hypertension (14), type 2 diabetes mellitus (11), chronic obstructive pulmonary disease (10), and ischemic heart disease (7). The median Acute Physiology and Chronic Health Evaluation II score was 19 (IQR 14–23). The median sequential organ failure assessment score at critical care admission was 8 (IQR 5–10). Table 1 describes illness severity parameters at each phase of infection. No patients were readmitted to critical care between latent and convalescent phases. Table 2 describes clinical outcomes in this patient cohort. Thirty-eight patients had positive microbiological samples (17 positive blood cultures, 9 bronchoalveolar lavage, 9 tracheal aspirate, 12 sputum, 9 urine, and 18 abdominal swabs). The most common organisms identified by blood culture were *Escherichia coli* (9), *S pneumoniae* (3), and *Klebsiella* species (2). The source of infection was respiratory in 26 patients, abdominal in 21, and urogenital in 7. In common with previous work (23), microbiological yield was lower for respiratory infection (16/26) compared with that for abdominal (16/21) and urogenital (6/7) infections, $P = 0.043$ (Kruskal–Wallis). In our cohort, 11/16 samples from patients with respiratory infection grew gram-positive organisms.

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TABLE 1. **Illness severity and required organ support in early, latent, and convalescent phases of infection**

Parameter	Early (n = 54)	Latent (n = 39)	Convalescent (n = 18)
SOFA score	8 (5–12)	2 (1–5)	0 (0–0)
Mechanical ventilation	35 (64.8%)	18 (46.2%)	0 (0%)
Cardiovascular support	36 (66.7%)	4 (10.3%)	0 (0%)
Renal replacement therapy	7 (13.0%)	3 (7.5%)	0 (0%)

The table demonstrates the change in SOFA score (median, IQR) in each phase of infection and the number (%) of patients who required mechanical ventilation, cardiovascular support (noradrenaline 31/36), and renal replacement therapy. A total of 54/54 early, 29/39 latent, and 0/18 convalescent phase blood samples were taken with patients on critical care wards. IQR indicates interquartile range; SOFA, sequential organ failure assessment.

Eleven healthy volunteers were recruited to this study (six males) with a median age of 21 (IQR 21–27). All of the healthy volunteers were of white ethnic origin; there were no smokers and median body mass index was 24.5 (IQR 22.1–28.3). The volunteers had no premorbid conditions (median Charlson Index score 0, IQR 0–0). Three samples of venous blood were taken at intervals matched to that of the critical care patients (i.e., time 0, 1 week later, and 3–6 months later).

Opsonophagocytosis assay: P4 peptide stimulated increased bacterial killing

Neutrophil bacterial killing was 54.9% higher in P4-stimulated cells compared with that in paired controls (24.13% vs. 15.57%, $P < 0.001$). There was no interaction between P4 peptide effect and phase of infection, such that stimulated neutrophils demonstrated increased bacterial killing (8.55%, $P < 0.001$) in early, latent, and convalescent infections (Fig. 1). Raw data and details of the mixed model regression analysis are provided in the Supplemental Digital Content, http://links.lww.com/SHK/A443 (opsonophagocytosis assay analysis). There was no difference between DEPC and M12-1 peptide control, 16.65% versus 14.78%, $P = 0.366$ (Fig. S3, Supplemental Digital Content, http://links.lww.com/SHK/A443). In the mixed model regression analysis, the source of infection (respiratory 26, abdominal 21, urogenital 7) was not associated with P4 peptide activity. In addition, smoking status did not impact upon phagocytic function in this cohort. However, phase of infection was independently associated with baseline bacterial killing and was significantly increased in the latent and convalescent phase samples compared with early phase samples. We found no significant difference in mean of neutrophil bacterial killing between patients with positive microbiology and patients with negative microbiology in early phase samples in either control (14.15% vs. 17.89%, $P = 0.208$) or P4 peptide–stimulated experiments (23.49% vs. 26.69%, $P = 0.273$), unpaired *t* tests. In an exploratory analysis, clinical laboratory white cell, neutrophil, and platelet counts were not associated with baseline neutrophil bacterial killing or response to P4 peptide in early phase samples (Tables S1 and S2, Supplemental Digital Content, http://links.lww.com/SHK/A443). This study was not adequately powered to formally investigate the effect of P4 on samples from healthy volunteers, but the results were consistent with the patient samples: P4 peptide leads to significantly increased mean of neutrophil bacterial killing in blood sample one (day 0: 37.7% vs. 27.9%, $P < 0.001$, CI 7.0 – 12.6), blood sample two (day 7: 34.4% vs. 24.5%, $P < 0.001$, CI 7.0–12.6), and blood sample three (month 3: 34.0% vs. 24.2% $P < 0.001$, CI 7.0–12.6), mixed model regression analysis. Thus, baseline (vehicle control) neutrophil activity was initially lower in patient samples before recovery toward healthy volunteer levels (early 15.5%, latent 20.1%, and convalescent 23.2%; Fig. 1).

TABLE 2. **Clinical outcome measures from critical care patient cohort (n = 54)**

Group	Independent variable	Median	IQR
Organ support (days)	Mechanical ventilation	6	0–10
	Cardiovascular support	7	0–15
Length of stay (days)	Critical care	9	5–16
	Hospital	20	11–40
Mortality	Critical care	14 (25.9%)	
	Hospital	18 (34.0%)	
	28 days	18 (34.0%)	

The table displays median and IQR values for organ support and length of stay variables. For mortality values, the table displays n (%). IQR indicates interquartile range.

Whole blood phagocytosis assay: P4 peptide stimulates increased reporter bead association and oxidation

P4 peptide stimulation significantly increased intraphagosomal bead association (Fig. 2A) and intraphagosomal bead oxidation (Fig. 2B) compared with paired vehicle control. Raw data and details of the mixed model regression analysis are provided in the Supplemental Digital Content, http://links.lww.com/SHK/A443 (whole blood phagocytosis assay: neutrophil–bead association analysis; and whole blood phagocytosis assay: neutrophil intraphagosomal reporter bead oxidation analysis). A significant interaction was found between duration of incubation and P4 peptide stimulation in both reporter bead association and oxidation analyses. In these mixed model regression analyses, source of infection (respiratory 21, abdominal 17, and urogenital 6) was not independently associated with P4 peptide activity but baseline (vehicle control) bead association and oxidation were significantly decreased in the latent and convalescent phases of infection. There was no significant difference in bead association or oxidation (area under the curve calculations) between patients with positive microbiology and patients with negative microbiology in early phase samples exposed to vehicle control and P4 peptide (unpaired *t* tests). Additionally, in a nonpowered cohort of healthy volunteers, treatment with P4 peptide stimulated increased neutrophil–bead association compared with vehicle control (Fig. S4, Supplemental Digital Content, http://links.lww.com/SHK/A443).

Neutrophil CD66b expression

Neutrophils with increased expression of CD66b are associated with an upregulated respiratory burst in samples isolated from patients with severe sepsis (24). Baseline CD66b was higher in early phase samples compared with that in latent phase samples (Fig. 3A) and this was associated with greater neutrophil–bead association (Fig. 3B). There was no difference

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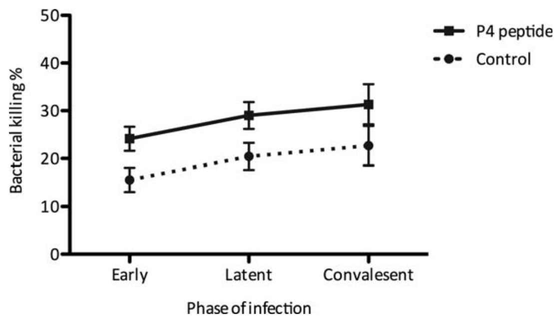

Fig. 1. **P4 peptide increases neutrophil bacterial killing in early, latent, and convalescent phases of infection (adjusted analysis).** The figure demonstrates the mixed model adjusted analysis for bacterial killing index (point estimate and 95% confidence interval) for neutrophils exposed to P4 peptide compared with that for vehicle (DEPC water) control in the opsonophagocytosis killing assay in patients admitted to critical care with severe sepsis. Killing index describes how the addition of neutrophils to complement and immunoglobulin opsonized *Streptococcus pneumoniae* decreases subsequent bacterial growth—calculated as follows: (1 – [colony-forming units experimental well])/[colony-forming units opsonized non-neutrophil control well]) × 100. The addition of P4 peptide increased the mean neutrophil bacterial killing compared with vehicle (DEPC water) control in early (24.2 vs. 15.5, $P < 0.001$), latent (28.7 vs. 20.1, $P < 0.001$), and convalescent (31.8 vs. 23.2, $P < 0.001$) phases of infection. DEPC indicates diethylpyrocarbonate.

in expression between samples exposed to P4 peptide and vehicle control.

Monocyte activity

Monocytes were identified by cell surface expression of CD14 in 51/94 of blood samples taken from patients for the whole blood phagocytosis assay. Seven samples identified <750 monocytes and were excluded from the analysis. P4 peptide–stimulated monocytes had significantly increased intraphagosomal reporter bead association in early, latent, and convalescent phases of sepsis by 8.3% (CI 3.4–13, $P = 0.001$). Monocyte oxidation ratio was significantly increased in samples exposed to P4 peptide compared with that in vehicle control (1.39 vs. 1.05, CI 0.23–0.44, $P < 0.001$). Raw data and details of the mixed model regression analysis are provided in the Supplemental Digital Content, http://links.lww.com/SHK/A443 (whole blood phagocytosis assay: monocyte-bead association analysis; and whole blood phagocytosis assay: monocyte intraphagosomal reporter bead oxidation analysis).

DISCUSSION

We found that augmented passive immunotherapy *ex vivo* using P4 peptide stimulation significantly increased neutrophil bacterial killing in samples from patients with severe sepsis.

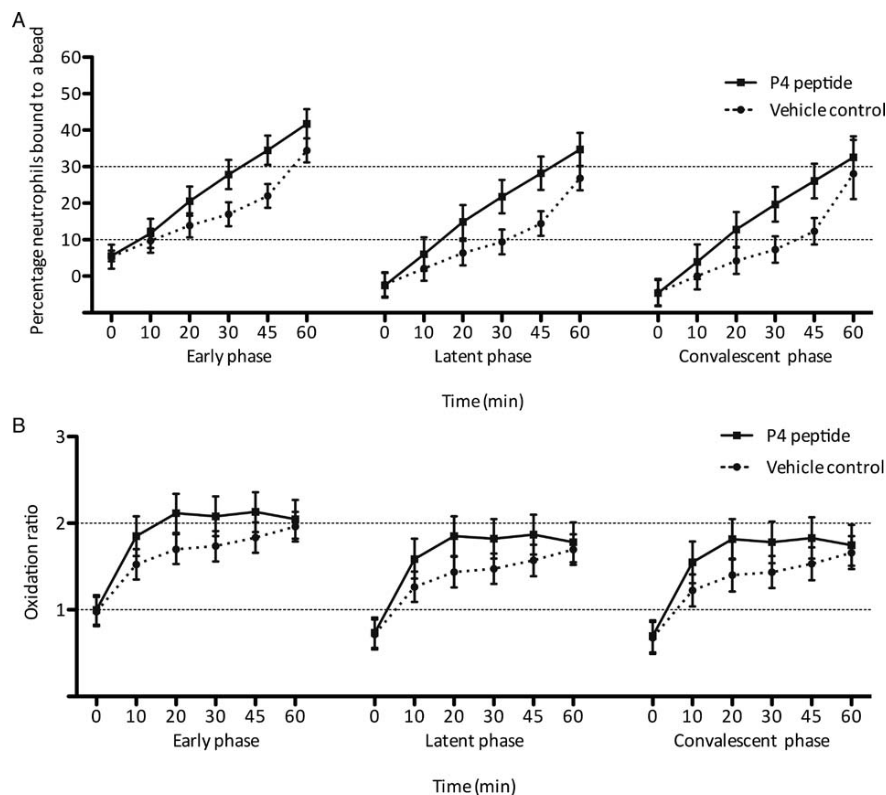

Fig. 2. **P4 peptide increases intraphagosomal reporter bead and oxidation association in early, latent, and convalescent phases of severe infection.** A, The mixed model adjusted mean proportion (95% confidence interval) of neutrophils associated with an intraphagosomal reporter bead at 0, 10, 20, 30, 45, and 60 min in the early, latent, and convalescent phases of infection. B, The mixed model adjusted mean oxidation ratio (95% confidence interval) of intraphagosomal reporter beads associated with neutrophils at 0, 10, 20, 30, 45, and 60 min in the early, latent, and convalescent phases of infection.

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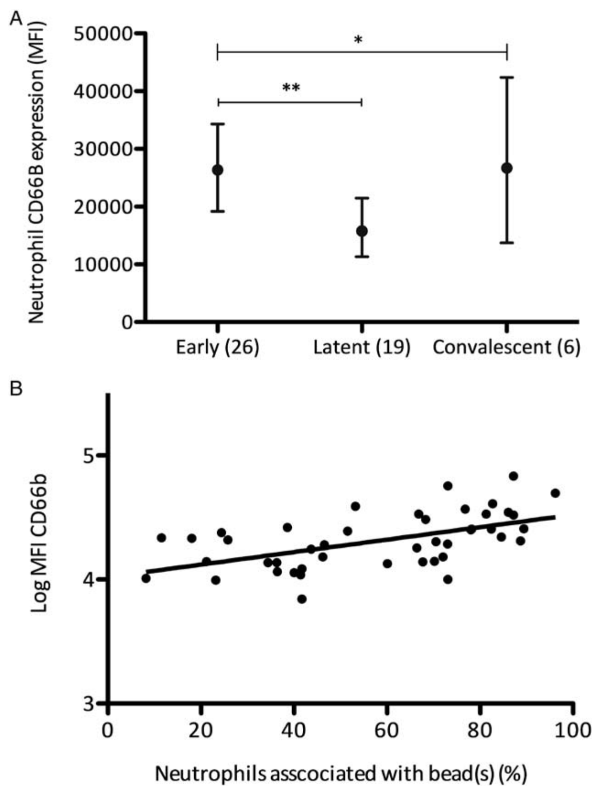

FIG. 3. **Neutrophil CD66b expression is lower in latent infection and significantly associated with intraphagosomal reporter bead association.** A, Neutrophil 66B expression (median, IQR) in early and latent phases of infection. There was a significant difference between the three phases of infection (H[2] = 8.43, $P = 0.015$), Kruskal–Wallis rank test. Mean fluorescence intensity was significantly decreased in blood samples taken in latent phase infection (median 15,200 vs. 25,411, $P = 0.002$) compared with that in blood samples taken in early phase infection in patients admitted to critical care; Mann–Whitney *U* test. B, Neutrophil association with beads after 45-min incubation compared with mean fluorescence intensity of cell surface 66B expression in early and latent phases of infection. Linear regression analysis demonstrated a significant association between log MFI and neutrophil–bead association (F[1, 45] = 18.93, $P < 0.001$, $R^2 = 0.306$). IQR indicates interquartile range.

This effect was independent of the source, microbiological status, and phase of infection. These data are corroborated by the whole blood phagocytosis assay that demonstrated P4-mediated increase in intraphagosomal reporter bead association and oxidation. This study demonstrates evidence that augmented passive immunotherapy has promise as a potential therapeutic strategy for patients with severe infection.

We recruited patients admitted to critical care with severe infection with a wide range of comorbidities, infecting pathogens, severity of illness, and age. Therefore, our cohort represents a broad spectrum of critically ill patients who could potentially benefit from treatment with augmented passive immunotherapy. Importantly, we have demonstrated a persistent response to P4 peptide in the latent phase of sepsis. Downregulation of the immune system leaves patients prone to secondary hospital-acquired infection, so this strategy of phagocyte stimulation may also benefit patients during later stages of disease (16).

Historically, adjuvant immunotherapies have failed to improve outcomes for patients with community-acquired pneumonia and sepsis (6). However, there is increasing focus on immunostimulatory therapies (25) for infection with recent work exploring the efficacy of interleukin-7 and programmed death ligand 1 (26) in modulating T-cell responses and granulocyte-macrophage colony-stimulating factor (NCT01653665) and interferon-γ (NCT01649921) in modulating innate immune responses. Our study supports the addition of augmented passive immunotherapy with P4 peptide to the list of potential immunostimulatory adjuvant therapies for severe infection. Treatment with augmented passive immunotherapy has previously been shown to rescue moribund septic mice either alone or in combination with antibiotics (9). The risk of immune hyperstimulation and organ injury is of concern for immunostimulatory therapies (27). However, P4 peptide does not stimulate inflammatory cytokine release *ex vivo* (15) and activated neutrophils can be retained and "deprimed" within the lungs (28). This mechanism of depriming is reduced for patients with adult respiratory distress syndrome; therefore, the potential detrimental effects of P4 peptide in this specific context require further investigation.

We used two robust methods to determine phagocytic activity. First, the opsonophagocytosis killing assay is an established and standardized method (29), widely used to compare phagocytic function in vaccine efficacy testing (30) and applied in previous work with P4 peptide (7, 8, 12, 15). As previously described (15), P4-mediated effects on phagocyte bacterial uptake depend on pathogen opsonization. Our study investigated *S Pneumoniae*, but a previous *in vitro* study has demonstrated that P4 peptide can increase phagocytic uptake of opsonized gram-positive and gram-negative bacteria (8). We used pooled human IVIG as a standardized opsonin in our opsonophagocytosis killing assay. Future clinical investigation could use P4 peptide administered with human IVIG as an empirical opsonin against a range of bacteria (6). We used a secondary whole blood phagocytosis assay to complement the opsonophagocytosis assay using silica beads opsonized with human immunoglobulin. The aims of this assay were to reduce sample preprocessing and attempt to maintain the influence of inflammatory mediators. We chose a kinetic assay (multiple incubation intervals) to determine how P4 peptide influenced both the magnitude and the timing of oxidative burst. Incubation intervals were based on previous work (21, 31) that demonstrates maximal oxidative burst at 30 min of exposure to the phagosome.

While both the opsonophagocytosis and whole blood phagocytosis assays demonstrated that P4 peptide stimulation augmented neutrophil activity, there were differences in baseline (nonstimulated) neutrophil activity at different phases of infection. Downregulation of the immune system and impaired neutrophil activity has been observed after an initial hyperimmune response to infection—characterized by the "compensatory anti-inflammatory response syndrome" model of immune activity (32). Our whole blood phagocytosis assay data demonstrated this effect, but the opsonophagocytosis assay did not (Figs. 1 and 2). We hypothesized that density isolation and purification of neutrophils in the opsonophagocytosis assay would preferentially select mature neutrophils (33) and discarded lower density, less mature cells. We therefore used

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neutrophil CD66b expression, a marker associated with integrin-mediated adhesion and generation of reactive oxygen species (24), as a surrogate for neutrophil maturity in our whole blood assay. In support of our hypothesis, we observed significantly decreased CD66b expression in the latent phase of infection and significant correlation between neutrophil expression and association with intraphagosomal reporter beads (Fig. 3). In addition, we observed decreased monocyte–bead association in the latent and convalescent phases of infection using our whole blood phagocytosis assay. These results, coupled with previous evidence (33), suggest that density isolation of neutrophils limited our ability to measure the compensatory anti-inflammatory response using the opsonophagocytosis assay. An alternative neutrophil purification technique (e.g., magnetic negative cell selection) could mediate for this effect but requires further investigation.

We used the M12-1 fragment of streptococcal group A *emm12* protein, a relevant immunogenic peptide control that has been described as a virulence factor for streptococcal group A infection (34) and induces adaptive immunogenic responses in mice (35). We found no increase in neutrophil bacterial killing using this control peptide so pragmatically used DEPC water as the vehicle control for the remainder of the study, as per previously published work (14, 15). For the whole blood phagocytosis assay we noted convergence of results after 60-min incubation between P4 peptide and vehicle control–stimulated samples. For oxidation ratio we highlighted the plateau in oxidation ratio observed using this method. Vehicle control samples reached this plateau but at a slower rate than samples stimulated with P4 peptide. There was also a reduced but still significant difference in neutrophil–bead association at 60 min; this could be due to neutrophil saturation (only a proportion of neutrophils will associate with beads) or digestion of beads within the phagolysome, but these hypotheses require further investigation. The purpose of this study was to measure cellular response to P4 peptide; as such, we did measure additional inflammatory mediators. Previous work demonstrates that P4 peptide stimulation does not influence cellular release of a number of inflammatory cytokines (15). Future work should seek to determine the influence of inflammatory mediators on P4 peptide activity.

There was a relatively high dropout for convalescent samples in our cohort. Mortality accounted for 18/36 of dropouts, but other patients were too unwell to attend or declined our invitation citing multiple other hospital appointments—this highlights the persistent morbidity often seen after critical illness. The purpose of the healthy volunteer cohort was to standardize study assays (healthy volunteer P4 peptide activity is previously published (15)). The small number of samples and differences in volunteer demographics (age, body mass index, and comorbidities) limit our ability to directly compare patient and volunteer samples and draw conclusions about the presence and onset of compensatory anti-inflammatory response syndrome in our patient cohort. In addition, the demographic variation of our study group was limited (52/54 patients were whites); this limits our ability to extrapolate our results to a more diverse cohort. Currently, this therapeutic strategy is subject to preclinical toxicology investigations in preparation for early phase clinical trials. This study will guide future clinical studies as we seek to further refine the target patient population.

CONCLUSION

In conclusion, augmented passive immunotherapy using P4 peptide significantly increased phagocytic killing in samples taken from patients admitted to critical care with severe infection. The microbiological opsonophagocytic assay was supported by a flow cytometric whole blood assay that demonstrated increased intraphagosomal reporter bead association and oxidation in both neutrophils and monocytes. P4 peptide boosted the innate immune response to infection, enhancing bacterial killing by key immune effector cell populations and demonstrating clear potential as a future treatment of the most common causes of severe infection.

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