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

Ben Morton,*† Elena Mitsi,† Shaun H. Pennington,‡† Jesús Reine,† Angela D. Wright,§ Robert Parker,† Ingeborg D. Welters,† John D. Blakey,† Gowrisankar Rajam,† Edwin W. Ades,† Daniela M. Ferreira,† Duolao Wang,† Aras Kadioglu,† and Stephen B. Gordon†

*Aintree University Hospital NHS Foundation Trust, Liverpool, UK; †Clinical Sciences, Liverpool School of Tropical Medicine, Liverpool, UK; ‡Clinical Infection, Microbiology and Immunology, Institute of Infection & Global Health, University of Liverpool, Liverpool, UK; §Local Comprehensive Research Network, Northwest Coast, Liverpool, UK; ††Institute of Ageing and Chronic Disease, University of Liverpool, Liverpool, UK; †Division of Bacterial Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia; and ‡Clinical Infection, Microbiology and Immunology, Institute of Infection & Global Health, University of Liverpool, Liverpool, UK

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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 (>3 months postdischarge). Blood samples were taken in early (<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
bacterial killing when exposed to P4 peptide \textit{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 \textit{in vitro} and \textit{ex 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 \textit{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 Antwerp 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 cause (18)). Patient exclusion criteria were as follows: immunocompromised condition or therapy, pregnancy, responsible clinician deemed inappropriate, co-enrolment 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 sampling 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 (21)). Briefly, neutrophils were isolated from heparinized blood by density centrifugation and incubated (45 min, 37°C, shaking 300 RPM) with live pneumococci (capsulated type 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 determined by a standardized opsonophagocytosis assay (with minor modifications (20)). Briefly, neutrophils were reconstituted and purified by DEPCtreated (30 $\times$ 10^6/mL) in Roswell Park Memorial Institute media (Invitrogen, USA) and mixed with experimental solution (4 mg/mL P4 peptide or plain DEPC). The dose was based on a P4 peptide titration experiment (Supplemental Digital Content, http://links.lww.com/SHK/A443). Subsequently, paired 120 $\mu$L aliquots (0, 10, 20, 30, 45, and 60 min) were incubated with whole blood (100 $\mu$L) at 37°C in the dark. Multiphoton 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 alloted incubation time, samples were rapidly cooled (4°C) to arrest biological activity, red cells treated with lysing 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 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 \textit{Escherichia coli} (9), \textit{S pneumoniae} (3), and \textit{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, $P = 0.043$ (Kruskall–Wallis). In our cohort, 11/16 samples from patients with respiratory infection grew gram-positive organisms.
 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% (Fig. S3, Supplemental Digital Content). 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).

### 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).

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Early (n = 54)</th>
<th>Latent (n = 39)</th>
<th>Convalescent (n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOFA score</td>
<td>8 (5–12)</td>
<td>2 (1–5)</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td>35 (64.8%)</td>
<td>18 (46.2%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Cardiovascular support</td>
<td>36 (68.7%)</td>
<td>4 (10.3%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Renal replacement therapy</td>
<td>7 (13.0%)</td>
<td>3 (7.5%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

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 0/16 convalescent phase blood samples were taken with patients on critical care wards. IQR indicates interquartile range; SOFA, sequential organ failure assessment.

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

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

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.

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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 \textit{ex vivo} using P4 peptide stimulation significantly increased neutrophil bacterial killing in samples from patients with severe sepsis.
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
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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**REFERENCES**


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