Shock, Publish Ahead of Print

DOI : 10.1097/SHK.0000000000000715

Augmented passive immunotherapy with P4 peptide improves phagocyte activity in severe sepsis.

Authors

Ben Morton[1,2]*, Elena Mitsi[2]*, Shaun H Pennington[2,3], Jesús Reiné[2], Angela D Wright[2,4], Robert Parker[1,2], Ingeborg D Welters[5], John D Blakey[1,2], Gowrisankar Rajam[6], Edwin W Ades[6], Daniela M Ferreira[2], Duolao Wang[2], Aras Kadioglu[7], Stephen B Gordon[2]

*Joint first authorship

1) Aintree University Hospital NHS Foundation Trust, Liverpool, UK
2) Clinical Sciences, Liverpool School of Tropical Medicine, Liverpool, UK.
3) Clinical Infection, Microbiology and Immunology, Institute of Infection & Global Health, University of Liverpool, Liverpool, UK
4) Local Comprehensive Research Network, Northwest Coast, Liverpool, UK
5) Institute of Ageing and Chronic Disease, University of Liverpool, UK
6) Division of Bacterial Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA
7) Clinical Infection, Microbiology and Immunology, Institute of Infection & Global Health, University of Liverpool, UK

Corresponding author: Dr Ben Morton: Clinical Sciences, CTID Building, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA. Tel: 0151 705 3100. Email: ben.morton@lstmed.ac.uk

Key words: neutrophil biology, innate immunity, bacterial infection.

Funding and support

This work was supported by funds from Liverpool Health Partners, University of Liverpool (2013 to SBG) and the Medical Research Council Confidence in Concept Scheme (2013 to SBG). These funding sources had no role in study design, in the collection, analysis and interpretation of data, in the writing of the report, or in the decision to submit the paper for publication.

Declaration of interests

BM has received an honorarium for a lecture related to P4 peptide presented to Grifols Inc. in 2014. The remaining authors declare no other competing interests.

Running Head:

P4 peptide augments phagocyte function in sepsis

Author contributions:

Substantial contributions to the conception or design of the work

BM, EM, SHP, ADW, RP, IDW, JDB, GR, EWA, DMF, AK, SBG

Acquisition of data

BM, EM, SHP, JR, ADW, RP

Analysis of data

BM, EM, SHP, JR, DMF, DW, SBG

Interpretation of data

BM, EM, SHP, JR, RP, IDW, JDB, GR, EWA, DMF, DW, AK, SBG

Drafting work for important intellectual content

BM, EM, SHP, JR, ADW, RP, IDW, JDB, GR, EWA, DMF, DW, SBG

Final approval of version to be published

BM, EM, SHP, JR, ADW, RP, IDW, JDB, GR, EWA, DMF, DW, AK, SBG

Accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

BM, EM, SHP, JR, ADW, RP, IDW, JDB, GR, EWA, DMF, DW, AK, SBG

Acknowledgements

The authors would like to acknowledge the support of the research nurses based at Aintree University Hospital and the Royal Liverpool Hospital for patient recruitment and prospective clinical data collection: Sister Colette Seasman, Sister Carole Hancock, Sister Karen Williams, Sister Anna Walker, Sister Victoria Waugh, Sister Julie Patrick-Hesselton, Sister Sam Hendry and Staff nurse David Shaw. We would also like to thank Dr Jamie Rylance, Dr Lorena Preciado-Llanes and Dr Kondwani Jambo for their support and advice in developing the whole blood phagocytosis assay.

ABSTRACT

Introduction: Antimicrobial resistance threatens to undermine treatment for severe infection; new therapeutic strategies are urgently needed. Pre-clinical 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 post discharge). Blood samples were taken in early (≤ 48hrs post-diagnosis, n=54), latent (seven days post-diagnosis, n=39) and convalescent (3-6 months post-diagnosis, n=18) phases of disease. The primary outcome measure was killing of opsonised *S.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 opsonised pneumococci by 8.6% (C.I. 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 to 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 pre-clinical 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.

INTRODUCTION

Severe sepsis is a worldwide public health concern despite recent advances and standardisation 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 *Streptococcus 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). Further, healthy human alveolar macrophages demonstrate improved 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.

MATERIALS 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 - June 2015). Patient inclusion criteria: age ≥18, recruitment ≤48hours diagnosis, severe sepsis from respiratory, abdominal or urogenital source (predominant causes (18)). Patient exclusion criteria were: immunocompromising condition or therapy, pregnancy, responsible clinician deemed inappropriate, co-enrolment in another study that could have influenced results or failed to obtain consent. Blood samples were taken at day 0, 7 and 3-6 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 volunteers inclusion were: 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.

Laboratory

P4 peptide and M12-1 peptide were synthesized and purified by Centers for Disease Control, Georgia, USA and reconstituted in diethylpyrocarbonate (DEPC) water. The primary outcome measure was neutrophil bacterial killing determined by a standardised opsonophagocytosis assay (with minor modifications (20)). Briefly, neutrophils were isolated from heparinized blood by

density centrifugation and incubated (45 minutes, 37^{o}C, shaking 300 RPM) with live pneumococci (capsulated serotype 2, D39 strain), human intravenous immunoglobulin (Gammunex, Grifols, Inc.), baby rabbit complement (Mast Group, UK) and P4 peptide (1mg/ml) or control (M12-1 peptide 1mg/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^{o}C) to arrest phagocytic activity and the mixture incubated for 18-24 hours (blood agar, 37^{o}C, 5% CO_2) before colony forming unit enumeration. All samples were performed in triplicate. Neutrophil bacterial killing index is defined as: 1 – (experimental well divided by non-PMN control well). Experimental components were: neutrophils (PMN), opsonised bacteria and complement with P4 peptide, M12-1 peptide or vehicle control. Non-PMN components were: opsonised bacteria, complement. See Supplemental Digital Content for full details of experimental methods.

Our whole blood phagocytosis assay built on previous work (21). After venepuncture, citrated blood (4.5ml, BD) was placed on an automated mixer at room temperature. The intraphagosomal reporter beads constitute IgG coated silica beads with calibrator (Pacific Blue, Invitrogen) and reporter (OxyBurst Green, Invitrogen) fluorochromes. Beads were reconstituted (30 x10^{6}/ml) in Roswell Park Memorial Institute media (Invitrogen, US) and mixed with experimental solution (4mg/ml P4 peptide or plain DEPC): 5:1 ratio. This dose was based on a P4 peptide titration experiment (supplemental digital content). Subsequently, paired 120µl aliquots (0, 10, 20, 30, 45 and 60 minutes) were incubated with whole blood (100µl) at 37^{o}C in the dark. Multiple fluorescence minus one controls were used to set negative and positive boundaries for analysis (Figure S2, Supplemental Digital Content). After allotted incubation time, samples were rapidly cooled (4^{o}C) to arrest biological activity, red cells treated with lysis buffer (Biolegend, US) and samples washed before flow cytometric acquisition. In preliminary work, cell surface markers were used to define monocyte populations (CD14, BD Pharmingen) and estimate neutrophil function (CD66b, Stemcell). Compensation beads (BD Biosciences) 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, 488 and 633nm, Becton Dickinson) using FacsDiva (version 6.1, BD Biosciences) and FlowJo (version 10.7, Tree Star) flow cytometry software.

Statistics

Data were examined for normality before appropriate tests applied. All patient samples were analysed 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 employed 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 95% 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 was analysed using STATA 13.1 (Statacorp).

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 (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). Pre-morbid status was

quantified using Charlson-Index score ((22) median 2, IQR 1-3). The most common pre-morbid conditions were: hypertension (14), type 2 diabetes mellitus (11), chronic obstructive pulmonary disease (10) and ischaemic heart disease (7). The median acute physiology and chronic health evaluation two score (APACHE II) was 19 (IQR: 14-23). The median sequential organ failure assessment (SOFA) 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), *Streptococcus 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 to abdominal (16/21) and urogenital (6/7), 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 (6 male) 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 pre-morbid 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 to paired controls (24.13% vs. 15.57%, $p<0.001$). The 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 infection (Figure 1). Raw data and details of the mixed model regression analysis are provided in the supplemental digital content (opsonophagocytosis assay analysis). There was no difference between DEPC and M12-1 peptide control 16.65% vs. 14.78%, p=0.366 (Figure 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 compared to early phase samples. We found no significant difference in mean of neutrophil bacterial killing between patients with positive microbiology compared to negative in early phase samples either in 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). 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 lead to significantly increased mean of neutrophil bacterial killing at blood sample one (day 0: 37.7% vs. 27.9%, p<0.001 C.I. 7.0 – 12.6), blood sample two (day 7: 34.4% vs. 24.5%, p<0.001 C.I. 7.0 – 12.6) and blood sample three (month 3: 34.0% vs. 24.2% p<0.001 C.I. 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% - Figure 1).

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

P4 peptide stimulation significantly increased intraphagosomal bead association (Figure 2A) and intraphagosomal bead oxidation (Figure 2B) compared to paired vehicle control. Raw data and

details of the mixed model regression analysis are provided in the supplemental digital content (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 analysis, 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 calculation) between patients with positive microbiology compared to negative in early phase samples exposed to vehicle control and P4 peptide, using unpaired t-tests. Additionally, in a non-powered cohort of healthy volunteers, treatment with P4 peptide stimulated increased neutrophil-bead association compared to vehicle control (Figure S4, Supplemental Digital Content).

Neutrophil CD66b expression

Neutrophils with increased expression of CD66b are associated with an up-regulated respiratory burst in samples isolated from patients with severe sepsis (24). Baseline CD66b was higher in early phase compared to latent phase samples (Figure 3A) and this was associated with greater neutrophil-bead association (Figure 3B). There was no difference in expression between samples exposed to P4 peptide or 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 less than 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% (C.I. 3.4 – 13%, p=0.001). Monocyte oxidation ratio was significantly increased in samples exposed to P4 peptide compared to vehicle control (1.39 vs. 1.05, C.I. 0.23 – 0.44, p<0.001). Raw data and details of the mixed model regression analysis are provided in the supplemental digital content (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. 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 co-morbidities, 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. Down-regulation 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 IL-7 and PD-L1 (26) in modulating T-cell responses and GM-CSF (NCT01653665) and interferon-gamma (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 'de-primed' within the lungs (28). This mechanism of de-priming is reduced for patients with adult respiratory distress syndrome (ARDS) 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 standardised 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 opsonisation. Our study investigated *Strep. pneumoniae* but previous a *in vitro* study has demonstrated that P4 peptide can increase phagocytic uptake of opsonised gram-positive and gram-negative bacteria (8). We used pooled human IVIG as a standardised opsonin in our opsonophagocytosis killing assay. Future clinical investigation could employ 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 compliment the opsonophagocytosis assay using silica beads opsonised with human immunoglobulin. The aims of this assay were to reduce sample pre-processing 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 timing of oxidative burst. Incubation intervals were based on previous work (21,31) that demonstrates maximal oxidative burst at 30 minutes of exposure to the phagosome.

Whilst both the opsonophagocytosis and whole blood phagocytosis assays demonstrated that P4 peptide stimulation augmented neutrophil activity, there were differences in baseline (non-stimulated) neutrophil activity at different phases of infection. Down-regulation of the immune system and impaired neutrophil activity has been observed after an initial hyper-immune response to infection – characterised 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 (Figures 1 & 2). We hypothesised 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 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 (Figure 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 minutes incubation between P4 peptide and vehicle control stimulated samples. For oxidation ratio we highlight 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 minutes; 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 drop out for convalescent samples in our cohort. Mortality accounted for 18/36 of dropout 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 standardise study assays (healthy volunteer P4 peptide activity is previously published (15)). The small number of samples and differences in volunteer demographics (age, BMI and co-morbidities) 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 white); this limits our ability to extrapolate our results to a more diverse cohort. Currently, this therapeutic strategy is subject to pre-clinical 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 demonstrates clear potential as a future treatment for the most common causes of severe infection.

References

1. Goal-Directed Resuscitation for Patients with Early Septic Shock. N Engl J Med. 2014 Oct 1;141001063014008.

2. Mouncey PR, Osborn TM, Power GS, Harrison DA, Sadique MZ, Grieve RD, et al. Trial of Early, Goal-Directed Resuscitation for Septic Shock. N Engl J Med. 2015 Mar 17;372(14):150317011022003.

3. Yealy DM, Kellum JA, Huang DT, Barnato AE, Weissfeld LA, Pike F, et al. A randomized trial of protocol-based care for early septic shock. N Engl J Med. 2014 May 1;370(18):1683–93.

4. Dellinger RP, Levy MM, Rhodes A, Annane D, Gerlach H, Opal SM, et al. Surviving sepsis campaign: international guidelines for management of severe sepsis and septic shock: 2012. Crit Care Med. 2013 Feb;41(2):580–637.

5. The antibiotic alarm. Nature. 2013 Mar 14;495(7440):141.

6. Morton B, Pennington SH, Gordon SB. Immunomodulatory adjuvant therapy in severe community-acquired pneumonia. Expert Rev Respir Med. 2014 Jun 5;1–10.

7. Rajam G, Phillips DJ, White E, Anderton J, Hooper CW, Sampson JS, et al. A functional epitope of the pneumococcal surface adhesin A activates nasopharyngeal cells and increases bacterial internalization. Microb Pathog. 2008 Mar;44(3):186–96.

8. Rajam G, Skinner J, Melnick N, Martinez J, Carlone GM, Sampson JS, et al. A 28-aa pneumococcal surface adhesin A-derived peptide, P4, augments passive immunotherapy and rescues mice from fatal pneumococcal infection. J Infect Dis. 2009 Apr 15;199(8):1233–8.

9. Melnick N, Rajam G, Carlone GM, Sampson JS, Ades EW. Evaluation of a novel therapeutic approach to treating severe pneumococcal infection using a mouse model. Clin Vaccine

Immunol. 2009 Jun;16(6):806–10.

10. Rajam G, Sampson J, Carlone GM, Ades EW. An augmented passive immune therapy to treat fulminant bacterial infections. Recent Pat Antiinfect Drug Discov. 2010 Jun;5(2):157–67.

11. Rajam G, Bangert M, Hammons GM, Melnick N, Carlone GM, Sampson JS, et al. P4 peptide therapy rescues aged mice from fatal pneumococcal sepsis. Clin Vaccine Immunol. 2010 Nov;17(11):1823–4.

12. Rajam G, Hammons GM, Carlone GM, Sampson JS, Ades EW. A Novel Innate Immune-Enhancement Strategy Combined with IVIG Rescues Mice from Fatal Staphylococcus aureus Septicemia. Int J Microbiol. 2011 Jan;2011:725483.

13. Weeks JN, Boyd KL, Rajam G, Ades EW, McCullers J a. Immunotherapy with a combination of intravenous immune globulin and p4 peptide rescues mice from postinfluenza pneumococcal pneumonia. Antimicrob Agents Chemother. 2011 May;55(5):2276–81.

14. Bangert M, Bricio-Moreno L, Gore S, Rajam G, Ades EW, Gordon SB, et al. P4-mediated antibody therapy in an acute model of invasive pneumococcal disease. J Infect Dis. 2012 May 1;205(9):1399–407.

15. Bangert M, Wright AK, Rylance J, Kelly MJ, Wright AD, Carlone GM, et al. Immunoactivating peptide P4 augments alveolar macrophage phagocytosis in two diverse human populations. Antimicrob Agents Chemother. 2013 Jul 1;(July):0–7.

16. Hotchkiss RS, Monneret G, Payen D. Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. Nat Rev Immunol. 2013 Dec;13(12):862–74.

17. Levy MM, Fink MP, Marshall JC, Abraham E, Angus D, Cook D, et al. 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. Crit Care Med. 2003 Apr;31(4):1250–6.

18. Levy MM, Dellinger RP, Townsend SR, Linde-Zwirble WT, Marshall JC, Bion J, et al. The Surviving Sepsis Campaign: results of an international guideline-based performance improvement program targeting severe sepsis. Crit Care Med. 2010 Feb;38(2):367–74.

19. Demaret J, Venet F, Friggeri A, Cazalis M-A, Plassais J, Jallades L, et al. Marked alterations of neutrophil functions during sepsis-induced immunosuppression. J Leukoc Biol. 2015 Jul 29;

20. Romero-Steiner S, Frasch CE, Carlone G, Fleck RA, Goldblatt D, Nahm MH. Use of opsonophagocytosis for serological evaluation of pneumococcal vaccines. Clin Vaccine Immunol. 2006 Feb;13(2):165–9.

21. VanderVen BC, Yates RM, Russell DG. Intraphagosomal measurement of the magnitude and duration of the oxidative burst. Traffic. 2009 Apr;10(4):372–8.

22. Charlson ME, Pompei P, Ales KL, MacKenzie CR. A new method of classifying prognostic comorbidity in longitudinal studies: development and validation. J Chronic Dis. 1987 Jan;40(5):373–83.

23. Morton B, Nagaraja S, Collins A, Pennington SH, Blakey JD. A Retrospective Evaluation of Critical Care Blood Culture Yield - Do Support Services Contribute to the "Weekend Effect"? PLoS One. 2015 Jan;10(10):e0141361.

24. Martins PS, Brunialti MKC, Martos LSW, Machado FR, Assunçao MS, Blecher S, et al. Expression of cell surface receptors and oxidative metabolism modulation in the clinical continuum of sepsis. Crit Care. 2008 Jan;12(1):R25.

25. Boomer JS, Green JM, Hotchkiss RS. The changing immune system in sepsis: Is individualized immuno-modulatory therapy the answer? Virulence. 2013 Sep 25;5(1).

26. Shindo Y, Unsinger J, Burnham C-A, Green JM, Hotchkiss RS. Interleukin 7 and Anti-Programmed Cell Death 1 Antibody Have Differing Effects to Reverse Sepsis-Induced

Immunosuppression. Shock. 2015 Jan 6;

27. Suntharalingam G, Perry MR, Ward S, Brett SJ, Castello-Cortes A, Brunner MD, et al. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. N Engl J Med. 2006 Sep 7;355(10):1018–28.

28. Summers C, Singh NR, White JF, Mackenzie IM, Johnston A, Solanki C, et al. Pulmonary retention of primed neutrophils: a novel protective host response, which is impaired in the acute respiratory distress syndrome. Thorax. 2014 Jul;69(7):623–9.

29. Romero-Steiner S, Libutti D, Pais LB, Dykes J, Anderson P, Whitin JC, et al. Standardization of an opsonophagocytic assay for the measurement of functional antibody activity against Streptococcus pneumoniae using differentiated HL-60 cells. Clin Diagn Lab Immunol. 1997 Jul;4(4):415–22.

30. Song JY, Moseley MA, Burton RL, Nahm MH. Pneumococcal vaccine and opsonic pneumococcal antibody. J Infect Chemother. 2013 Jun;19(3):412–25.

31. Yates RM, Hermetter A, Russell DG. The kinetics of phagosome maturation as a function of phagosome/lysosome fusion and acquisition of hydrolytic activity. Traffic. 2005 May;6(5):413–20.

32. Hotchkiss RS, Karl IE. The pathophysiology and treatment of sepsis. N Engl J Med. 2003 Jan 9;348(2):138–50.

33. Drifte G, Dunn-Siegrist I, Tissières P, Pugin J. Innate immune functions of immature neutrophils in patients with sepsis and severe systemic inflammatory response syndrome. Crit Care Med. 2013 Mar;41(3):820–32.

34. Metzgar D, Zampolli A. The M protein of group A Streptococcus is a key virulence factor and a clinically relevant strain identification marker. Virulence. Jan;2(5):402–12.

35. Bruner M, James A, Beall B, Carlone GM, Ades E, Johnson S, et al. Evaluation of synthetic,

M type-specific peptides as antigens in a multivalent group A streptococcal vaccine.

Vaccine. 2003 Jun 20;21(21-22):2698–703.

Figure Legends

Figure 1: P4 peptide increases neutrophil bacterial killing in early, latent and convalescent phases of infection (adjusted analysis). Figure demonstrates the mixed-model adjusted analysis for bacterial killing index (point estimate and 95% confidence interval) for neutrophils exposed to P4 peptide compared to 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 opsonised *Streptococcus pneumoniae* decreases subsequent bacterial growth - calculated as: (1 – (colony forming units experimental well) / (colony forming units opsonised non-neutrophil control well)) x 100. The addition of P4 peptide increased the mean neutrophil bacterial killing compared to 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.

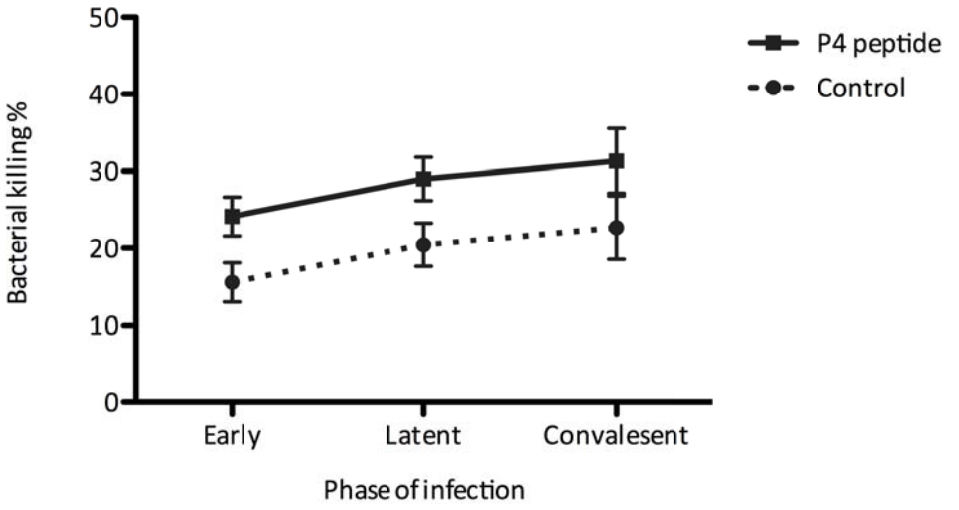


Figure 2: P4 peptide increases intraphagosomal reporter bead and oxidation association in early, latent and convalescent phases of severe infection. A: Figure displays the mixed-model adjusted mean proportion (95% confidence interval) of neutrophils associated with an intraphagosomal reporter bead at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

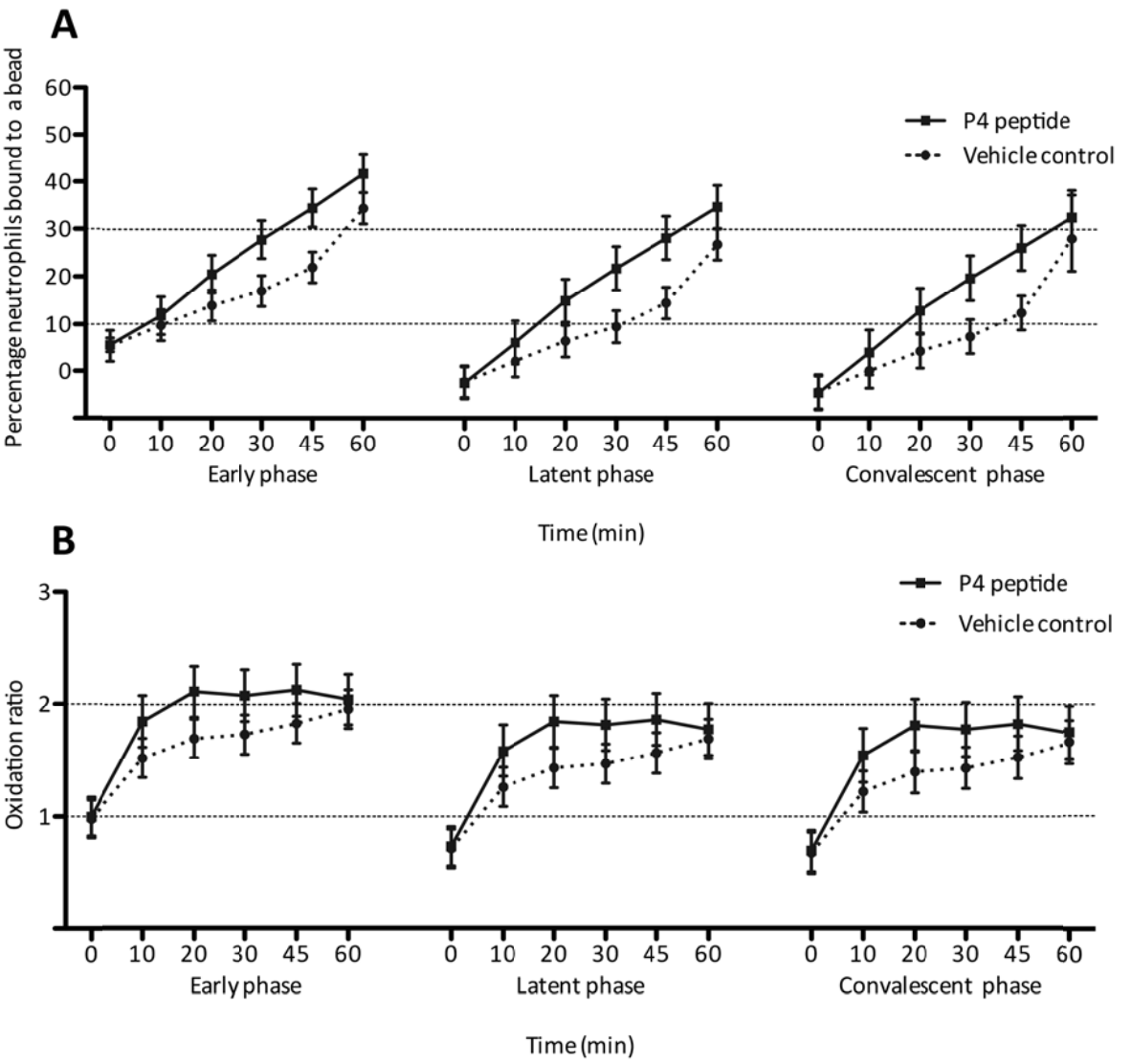


Figure 3: Neutrophil CD66b expression is lower in latent infection and significantly associated with intraphagosomal reporter bead association. A: Figure displays neutrophil 66B expression (median, IQR) in early and latent phases of infection. There was a significant difference between the three phases of infection (H[2]=8.43, p=0.015), Kruskall Wallis rank test. MFI was significantly decreased in blood samples taken in latent (median 15200 vs. 25411 p=0.002) compared to early phase infection in patients admitted to critical care; Mann-Whitney U Test. B: Figure displays neutrophil association with beads after 45 minutes incubation compared to 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).

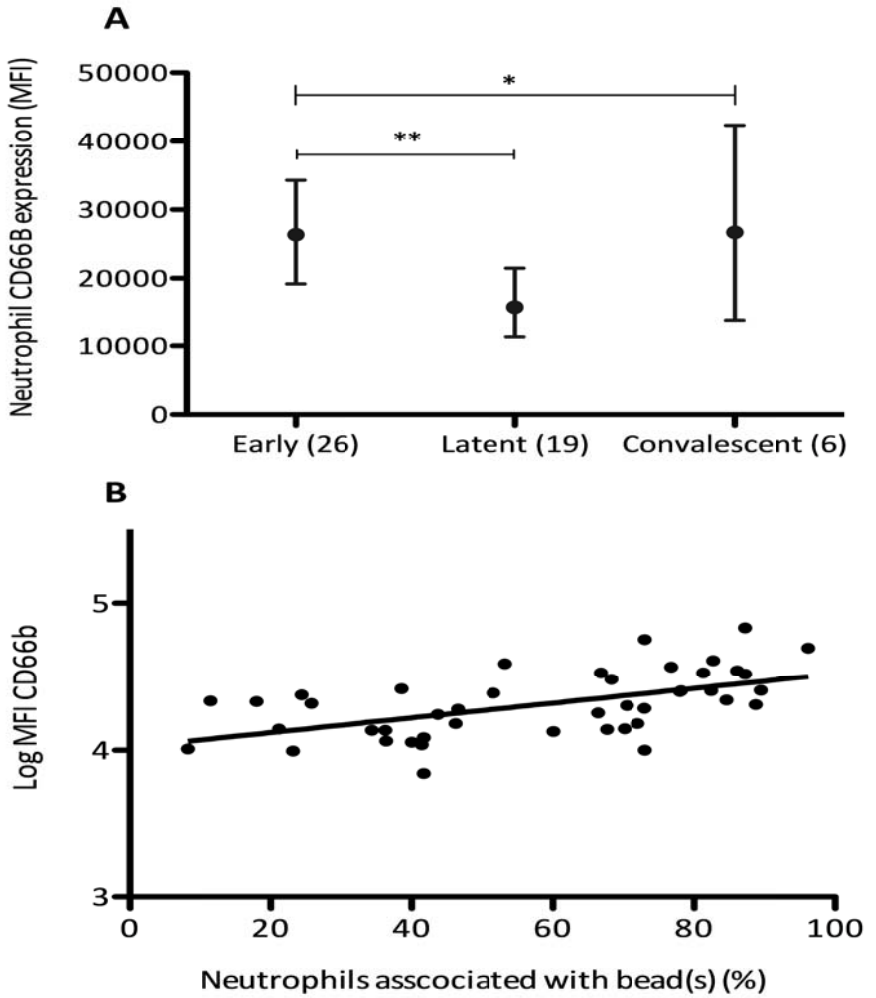


Table 1

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%)

Table 1: Illness severity and required organ support in early, latent and convalescent phases of infection. Table demonstrates the change in sequential organ failure assessment 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. 54/54 early, 29/39 latent and 0/18 convalescent phase blood samples were taken with patients on critical care wards.

Table 2

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 day	18 (34.0%)	

Table 2: Clinical outcome measures from critical care patient cohort (n=54). Table displays median and interquartile range values for organ support and length of stay variables. For mortality values table displays n (%).

Supplemental Digital Content

Methods

Trial design and participants

We prospectively recruited patients with severe sepsis who required admission to critical care. Sepsis, severe sepsis and septic shock were defined according to American College of Chest Physician - Society of Critical Care Medicine (ACCP-SCCM) criteria [1]. Briefly, sepsis is an infection that causes systemic inflammatory response syndrome (SIRS), severe sepsis is sepsis with end organ dysfunction and septic shock is sepsis with acute circulatory failure. Patients were recruited from critical care units at two university teaching hospitals: Aintree University Hospital NHS Foundation Trust and the Royal Liverpool University Hospital between February 2014 and June 2015; the last sample was taken in September 2015. Patient inclusion criteria were: age >18, recruitment within 48 hours of diagnosis, presence of severe sepsis (defined as above) and respiratory, abdominal or urogenital source of infection (predominant causes in critical care [2]). Patient exclusion criteria were: specific immunocompromising condition or therapy (e.g. chemotherapy), pregnancy, deemed inappropriate by responsible clinician (e.g. life expectancy < 48 hours), enrolment in an interventional study that might influence study results and failure to obtain consent. Blood samples were taken at day 0, 7 and after 3-6 months to measure neutrophil and monocyte activity in early, latent and convalescent phases of sepsis. Healthy volunteers were also recruited at the Clinical Research Facility, Royal Liverpool University Hospital and blood sampled at matched intervals. Inclusion criteria for healthy volunteers were: no current illness, age>18 and able to travel to the research facility. The North Wales NHS Research Ethics Committee approved this study on the 18th November 2013 (13/WA/0353). Written consent was obtained for all participants either directly or by a nominated consultee if incapacitated. Subsequently, written retrospective consent was obtained from patients that recovered. A CONSORT diagram details patient screening and inclusion for this study (Figure S1).

Clinical data

We prospectively recorded patient demographic data, physiological variables, clinical laboratory results, microbiology data, organ support, outcome and markers of illness severity including Acute Physiology and Chronic Health Evaluation II (APACHE II) and Sequential Organ Failure Assessment (SOFA). Data collection was completed in September 2015. Pragmatic clinical outcome measures were selected *a priori* to determine if output from the laboratory assays could predict clinically relevant measures. These were: ventilator dependent days [3], cardiovascular support days, intensive care length of stay, ICU mortality and 28 day mortality. In addition, clinical factors were selected *a priori* to determine association with assay results. These were: age, gender, need for mechanical ventilation, cardiovascular support or renal replacement therapy, APACHE II score, SOFA score, Charlson Index, P/F ratio, lactate, white cell count, neutrophil count, platelet count, creatinine level, and bilirubin level.

Laboratory

Peptide synthesis: P4 peptide as described was synthesised with the sequence LFVESSVKRRPMKTVSQDTNIPIYAQIF. The peptide was synthesized and purified at the Centre for Disease Control facility, Atlanta, Georgia, USA. An Advanced ChemTech 396 multiple peptide synthesizer was used to synthesize P4 using standard and modified 9-fluorenyl-methoxycarbonyl (Fmoc) protocols [4–6] resulting in a free N- and C-terminus. P4 peptide batches were prepared at >97% purity (measured by high-pressure liquid chromatography, HPLC), transported on dry ice to the UK and then stored at -20^{o}C until the day of use. For each sample peptide was solubilized in diethylpyrocarbonate (DEPC) water (1mg/ml OPKA and 4mg/ml whole blood assay) and stored at 4^{o}C until immediately before addition to assays. M12-1 is a peptide fragment of Streptococcal Group A *emm12* protein (synthesised as above by Centres for Disease Control, US) and this was used as a peptide control. M12-1 peptide is a well described virulence factor in Streptococcal Group A infection [7] and induces immunogenic responses in mice but not augmented phagocytic

responses [8]. Diethylpyrocarbonate (DEPC) water was used as solvent for the experimental solutions: 1mg/ml P4 peptide, 1mg/ml M12-1 peptide or plain DEPC water.

Opsonophagocytosis killing assay (OPKA)

The primary outcome measure for this study was neutrophil bacterial killing determined by a standard opsonophagocytosis assay with minor modifications [9]. Neutrophils from patient samples were isolated and incubated with live pneumococci, opsonins and either immunostimulatory peptide P4 or controls as below:

Bacterial culture: Capsulated serotype 2 (D39 strain) pneumococci were grown in Todd Hewitt broth supplemented with 0.5% yeast extract (THY) at 37^{o}C 5% CO_2 until optical density 0.3-0.4 was reached, measured at 600nm. After the pause of the liquid culture, bacteria were centrifuged at 13,000rpm for 15min, broth removed and bacteria pellet resuspended in THY 10% glycerol, aliquoted and stored at -80^{o}C. Bacterial concentration (CFU/ml) was quantified and standardised using the Miles and Misra technique [10].

Neutrophil isolation: Briefly, heparinised whole blood was separated by density centrifugation (with Histopaque® 1077 and 1119 at room temperature) into serum, peripheral blood mononuclear cells, polymorphonuclear leukocytes (PMNL) and red blood cells. The PMNL layer was removed and red blood cell contaminants lysed (Biolegend, US) prior to enumeration on a haemocytometer. Viability was assessed using Trypan blue (<90% viability excluded). PMNLs were then diluted in opsonisation buffer (Hanks Balanced Salt Solution with calcium and magnesium mixed with 1% gelatin at a 9:1 ratio) to a concentration of 1.7 x 10^6 PMNL/ml immediately prior to use in the opsonophagocytosis assay.

Bacterial opsonisation: For each assay an aliquot of standardised solution frozen D39 (ST2) bacteria was thawed and serially diluted to a concentration of 5 x 10^4 CFU/ml. Human intravenous antibody (Gammunex, Grifols, Inc.) was used as the opsonin, diluted to a 1 in 4 concentration with Hanks Balanced Salt Solution with magnesium and calcium ($HBSS^{+/+}$). An opsonised bacterial suspension was prepared by adding 500µL of diluted IVIG to 500µL of the bacterial suspension in a clear bijoux tube. A non-opsonised bacterial suspension was was prepared by adding 500µL $HBSS^{+/+}$ to 500µL of the bacterial suspension in a clear bijoux tube. Both bijoux tubes were then incubated at 37^{o}C for 20 minutes on a shaking incubator (100 RPM).

Opsonophagocytosis assay: Twenty microliters of the opsonised pneumococcal solution (500 bacteria), 10µL of baby rabbit complement (Mast Group, Merseyside UK) and 20µL of the experimental solution (peptide P4, control peptide or DEPC water control) were added in triplicate to a 96 well plate. Finally, 30µl of the 1.7 x 10^6 PMNL/ml/well solution (51,000 PMNL) was added to the solution. The result was a final volume of 80µl with a multiplicity of infection (MOI) 100:1. Appropriate controls wells were used with non-opsonised bacteria, heat inactivated complement (incubated at 56^{o}C for 30minutes), plain opsonisation buffer and plain DEPC water also added in triplicate to a 96 well plate. The 96 well plate was then covered and incubated at 37^{o}C for 45minutes with shaking (300 RPM). Subsequently, the plate was placed on ice for 15minutes to arrest the phagocytic process. After incubation, 10µL of the opsonophagocytic solution was taken from each well with a multiplex pipette and plated onto blood agar.

Colony forming unit enumeration: Blood agar plates were incubated at 37^{o}C with 5% CO_2 for 18-24 hours. The number of colonies forming units that grew from each 10µL were then counted the mean of the triplicate calculated. Neutrophil bacterial killing index was calculated as: 1 – (experimental well divided by non-PMN control well). The experimental well components were: isolated PMNLs, opsonised bacteria and complement with either P4 peptide, M12-1 peptide (12

samples) or vehicle control. The non-PMN control well components were: opsonised bacteria and complement. The effect of P4 was calculated as the absolute difference between the paired killing indices.

Whole blood phagocytosis assay (WBPA)

The whole blood phagocytosis assay was used to investigate how stimulation with P4 peptide influenced neutrophil association with intraphagosomal reporter beads and their subsequent oxidation within the phagosome. This assay builds upon previously published work using intraphagosomal reporter beads [11]. This assay was performed in parallel with the opsonophagocytosis assay from the same patient sample.

Intraphagosomal reporter bead manufacture: Carboxymethylated silica beads (Kisker Biotech, Germany - 3µm diameter) were incubated for 20 minutes with a N,N'-dicyclohexylcarbodiimide solution (Sigma, US) and subsequently washed and pelleted. These carbodiimide linked beads were then added to a coupling solution (sodium borate 0.1M pH 8.0), defatted bovine serum albumin (Sigma, US) and human immunoglobulin (IgG, Sigma, US) and incubated for 16hours. Beads were then washed and pelleted. IgG labelled beads were then added to a solution of reporter fluorochrome (OxyBurst Green, FITC, Invitrogen) and dimethylsulfoxide (DMSO) and incubated for 1 hour. The beads were then washed and this step once. Subsequently the beads were coupled to the calibrator Alexa fluor 405 (Pacific Blue, Invitrogen) using the two-step process as above. The IgG and fluorophore labelled beads were then washed, suspended in 0.02% sodium azide solution and divided into 90µl aliquots. Subsequently, beads were stored in the dark at -20^{o}C until use.

Assay constituents: Materials for the bead assay were defrosted and prepared on the day of blood sampling. One aliquot per blood sample of intraphagosomal reporter beads was washed in 1ml of Roswell Park Memorial Institute (RPMI) media (Invitrogen, US) and pelleted using a 30s spin at

13.3xG. This process was repeated three times to remove the sodium azide. Subsequently, beads were resuspended in a solution of 1.8ml RPMI (30 x 10^6 beads /ml). Experimental solutions were prepared (P4 peptide 4mg/ml, DEPC water and LPS 100ng/ml). Lipopolysaccharide (LPS) was used as a positive control with pre-made aliquots defrosted on the day of use (100ng/ml stored at -80^{o}C). Subsequently, aliquots of beads were mixed with the experimental solutions (10:2 parts). The P4 and DEPC solutions were then subdivided into 120µl aliquots for each incubation time point (0, 10, 20, 30, 45 and 60 minutes). One LPS solution was made for 45 minutes incubation. Samples were stored in the dark at room temperature prior to addition of whole blood at specified time points - see below.

Incubation and processing: After venepuncture, citrated blood (4.5ml 9NC (BD)) was placed on an automated mixer to prevent settling and permitted to reach room temperature. Subsequently, 100µl of whole blood was added to the paired bead solutions at pre-defined time intervals (60, 45, 30, 20, 10 and 0 minutes), commencing with the 60-minute sample. Blood and bead solutions were mixed with the pipette and then placed in a rotating incubator (300RPM) in the dark at 37^{o}C. The blood tube was placed on the mixer between time intervals. After 60 minutes all tubes were placed on ice in the dark for 10 minutes to arrest biological activity (0 minute tubes placed directly on ice). Subsequently, 2ml of red cell lysis buffer (5ml 10X RBC lysis buffer, Biolegend, US diluted in 45ml distilled water at 4^{o}C) was added to each tube and the experimental solution resuspended. The tubes were then placed at 4^{o}C for 20 minutes to permit red cell lysis. Following the lysis step, the tubes were centrifuged at 1300rpm for 5 minutes with brake. The supernatant was then poured away to leave the cell pellet. The pellet was then resuspended and washed in 2ml Dulbecco's phosphate-buffered saline (DPBS$^{-/-}$), no calcium and no magnesium. The centrifugation step was then repeated and supernatant poured away to leave a straw coloured pellet. The pellet was then resuspended in 300µL DPBS$^{-/-}$ and stored on ice, in the dark, until flow cytometric acquisition (within 2 hours).

Fluorescence Minus One (FMO) controls: RPMI was substituted for intraphagosomal reporter beads to exclude cellular autofluorescence after 0 and 45 minutes incubation with P4 peptide and vehicle control solutions. RPMI was substituted for blood and plain silica beads added to bead controls. Beads were incubated for 45 minutes to exclude independent intraphagosomal bead degradation/oxidation with P4 peptide and vehicle control solutions. These biological comparison controls were used to set negative and positive boundaries for flow cytometric analysis.

Cell surface markers: Cellular expression of CD14 (monocyte marker) and CD66B (neutrophil marker) were measured in a subset of samples. CD66b antibody was used to measure neutrophil cell surface expression of carcinoembryonic antigen gene family member 6 (CGM6). To do this, we evaluated an additional paired (vehicle control vs. P4 peptide) assay at 45 minutes with cell surface markers applied prior to acquisition. Samples that identified less than 750 monocytes were excluded from the analysis. The antibodies used were: PerCP-Cy 5.5 anti-human CD14 (clone MΦP9, mouse IgG2b, BD Pharmingen) and APC anti-human CD66b (clone G10F5, mouse IgMκ, Stemcell technologies).

Flow cytometric acquisition and analysis: Compensation beads (BD Biosciences) were used to create compensation matrices and a sequential cell gating strategy used to identify populations of interest. All samples were acquired using a BD LSRII Flow Cytometer equipped with three lasers emitting at 405, 488 and 633nm (Becton Dickinson) using FacsDiva (software version 6.1, BD Biosciences) and FlowJo (software version 10.7, Tree Star) flow cytometry software. At least 50,000 events were acquired per sample within a gate targeting the neutrophil population (according to FSC-A and SSC-A parameters).

For the whole blood phagocytosis assay the gating strategies are outlined in Figure S2. Neutrophil and intraphagosomal reporter bead association was determined as the percentage of gated (FSC-A,

SSC-A) neutrophils that expressed intraphagosomal reporter bead calibrator fluor. Monocyte and intraphagosomal reporter bead association was determined as the percentage of gated (CD14 positive) monocytes that expressed calibrator fluorochrome. Intraphagosomal reporter bead oxidation was calculated as a ratio of reporter fluorochrome mean fluorescence intensity (MFI, FITC) divided by calibrator fluorochrome MFI (Pacific Blue) in neutrophils and monocytes associated with beads.

Statistical analysis

Power calculations for this study were based on preliminary work that examined P4 peptide in patients with severe community acquired pneumonia (unpublished). The primary outcome was neutrophil mediated bacterial killing (%). P4 enhanced neutrophil bacterial killing from a mean (SD) of 30.78 (23.28) to a mean (SD) of 46.80 (12.79). Using these data we determined that recruitment of 58 patients would provide 90% power (alpha level <0.05, two tailed) to detect a 16% increase in bacterial killing.

Data were summarized using median (IQR), examined for normality and appropriate statistical tests applied. Linear mixed-model regression analyses were applied to the data to adjust for repeated measures in individual patients and determine if phase or source of infection were independent predictors of response to P4 peptide stimulation. In addition, for early phase samples, area under the curve was calculated using a statistical pharmacokinetic function (pksumm) to summarize output from the whole blood phagocytosis assay (all time points: 0, 10, 20, 30, 45 and 60 minutes) to determine association with clinical parameters (Figure S5). Linear and logistic regression analyses were performed to examine association between potential clinical predictors of assay output and to examine if the assay predicted clinical outcome measures. The effect of P4 was calculated as the absolute difference between the curves. Statistical analysis was performed using STATA 13.1, Statacorp, 2013.

Results

P4 peptide concentration in whole blood phagocytosis assay

The starting point for P4 peptide concentration was that used for the opsonophagocytosis assay. For this assay, 20µl 1mg/ml P4 peptide in DEPC water was added to the bacterial, neutrophil and complement solutions to constitute a total volume of 80µl per well (250µg/ml P4 peptide in solution). Therefore, using the equation $V_1 \times C_1 = V_2 \times C_2$, 20µl of 2.75mg/ml P4 peptide in DEPC was added to the blood-bead solution to (total volume of 220µl). However, in validation experiments, no difference between vehicle control and P4 peptide stimulated samples was observed at this concentration. Therefore, a titrated concentration experiment was used to determine optimal dose for this assay (Table S1). For this experiment, blood from a healthy volunteer was separated into aliquots and incubated with intraphagosomal reporter beads for 30 minutes with increasing doses of P4 peptide. Based on this work 4mg/ml P4 peptide in 20µl DEPC (final concentration 364µg/ml) was used for the finalised assay.

P4 peptide concentration	Neutrophil-bead association	Bead oxidation ratio
0 mg/ml	8.4%	1.12
2.75 mg/ml	8.6%	1.18
4 mg/ml	17.5%	1.38
5 mg/ml	14.3%	1.30
10 mg/ml	9.1%	1.36

Table S1: Illustrates results from a P4 peptide titration experiment to determine optimal dose for the whole blood phagocytosis assay. Citrated blood samples 100µl were incubated with intraphagosomal reporter beads (30×10^6 beads/ml in RPMI, 100µl) and 20µl of experimental solution (concentration as above) for 30 minutes. Samples were then lysed, processed and acquired by flow cytometry. Neutrophils were gated according to FSC-A and SSC-A parameters. Neutrophil-

bead association was defined as the proportion of neutrophils that expressed intraphagosomal reporter bead calibrator fluor (Pacific blue). Intraphagosomal reporter bead oxidation ratio was defined as mean fluorescence intensity reporter fluor (FITC) divided by calibrator fluor (Pacific Blue).

Association between blood parameters and baseline neutrophil bacterial killing

Group	Independent variable	Coef.	Std.Err.	p-value	R^2
Blood parameters	White Cell Count	-0.14	0.17	0.402	
	Neutrophils	-0.15	0.18	0.411	
	Platelets	-0.002	0.01	0.854	
	Creatinine	-0.02	0.01	0.116	
	Bilirubin	-0.005	0.03	0.844	

Table S1: Univariate regression analyses to determine association between patient clinical factors and baseline neutrophil bacterial killing. Table demonstrates the relationship between clinical factors (independent variables) and neutrophil bacterial killing calculated as (1 – (colony forming units experimental well) / (colony forming units opsonised non-neutrophil control well)) x 100. Coef. = coefficient. Std.Err. = standard error. Linear regression analysis.

Association between blood parameters and P4 peptide activity in the opsonophagocytosis killing assay

Group	Independent variable	Coef.	Std.Err.	p-value	R^2
Blood parameters	White Cell Count	0.13	0.1	0.233	
	Neutrophils	0.14	0.12	0.258	
	Platelets	-0.002	0.007	0.790	

	Creatinine	0.02	0.008	0.004	0.151
	Bilirubin	0.009	0.02	0.591	

Table S2: Univariate regression analyses to determine association between patient clinical factors and response to P4 peptide in neutrophil bacterial killing. Table demonstrates the relationship between clinical factors (independent variables) and absolute difference in bacterial killing calculated between neutrophils exposed to P4 peptide and vehicle control. Killing index is calculated as: (1 – (colony forming units experimental well) / (colony forming units opsonised non-neutrophil control well)) x 100. Coef. = coefficient. Std.Err. = standard error. Linear regression analysis.

Opsonophagocytosis assay analysis

Raw, non-adjusted, data from the opsonophagocytosis killing assay is presented. This data was used to generate the mixed-model regression analysis to adjust for the hierarchical nature of sampling (one patient sampled three times).

Opsonophagocytosis assay: raw data

Sample	**n**	**Control**	**C.I.**	**P4**	**C.I.**
Early	54	15.26	12.56 – 17.96	24.44	21.78 – 27.09
Latent	39	21.08	18.14 – 24.03	28.72	25.21 – 32.23
Convalescent	18	23.87	17.44 – 30.30	32.99	26.16 – 39.81

The neutrophil bacterial killing index is significantly higher in patient samples stimulated with P4 peptide in the early, latent and convalescent phases of severe infection. Table demonstrates mean and 95% confidence intervals (C.I.) for neutrophil bacterial killing index in samples stimulated with control (DEPC water) compared to P4 peptide (250μg/ml). This raw data was used for the mixed model regression analysis described in Figure 1 of the main manuscript. Statistical analysis by paired t-test.

The table below demonstrates the output from the mixed model regression analysis for patient opsonophagocytosis assay outcomes. Coefficients from this analysis were used in linear comparison analyses to generate point estimates and 95% confidence intervals used to generate Figure 1 in the main manuscript.

Opsonophagocytosis assay: adjusted data

Variable	Coefficient	P value	C.I.
Latent phase (vs. early)	4.88	<0.001	2.39 – 7.36
Convalescent phase (vs. early)	7.13	<0.001	3.23 – 11.04
P4 peptide (vs. control)	8.55	<0.001	6.35 – 10.76
Constant	15.54	<0.001	12.98 – 18.09

Random-effects parameters	Estimate	Std. Err.	C.I.
Individual variance	6.34	0.92	4.77 – 8.43
Residual variance	8.13	0.44	7.28 – 9.10

Mixed-model regression analysis for the opsonophagocytosis assay. Table illustrates significant factors that influence neutrophil bacterial killing in the opsonophagocytosis killing assay. Bacterial

killing index is calculated as: (1 – (colony forming units experimental well) / (colony forming units opsonised non-neutrophil control well)) x 100). The constant value represents the bacterial killing index by neutrophils from patients admitted to critical care with respiratory infection in the early phase of infection when exposed to vehicle control. Number of observations = 210, number of groups = 54, average observations per group = 3.9 (min 2, max 6). Individual variance refers to random effects at the patient level (level two error). Residual variance refers to level one error variance (variance of the overall error term). The coefficient describes the mean difference between groups. C.I. = confidence interval, Std. Err. = standard error.

Whole blood phagocytosis assay: neutrophil-bead association analysis

Raw, non-adjusted, data from the whole blood phagocytosis assay neutrophil-bead association output is presented below. This data was used to generate the mixed-model regression analysis to adjust for the hierarchical nature of sampling (one patient sampled three times).

The following table details the mixed-model regression analysis for neutrophil-bead association, including the interaction between P4 peptide and incubation period. . Coefficients from this analysis were used in linear comparison analyses to generate point estimates and 95% confidence intervals used to generate Figure 2A in the main manuscript.

Whole blood phagocytosis assay neutrophil-bead association: raw data

Sample	n	Incubation	Control	C.I.	P4	C.I.
Early	**44**	0	1.60	0.27 – 2.94	1.31	0.14 – 0.53
		10	7.87	5.64 – 10.10	11.68	8.37 – 14.99
		20	14.24	10.49 – 17.99	23.49	18.84 – 28.14
		30	17.70	13.16 – 22.25	32.07	26.07 – 38.08
		45	22.38	17.55 – 27.21	37.01	31.15 – 42.87
		60	34.65	28.79 – 40.52	43.51	37.30 – 49.71
Latent	**32**	0	0.60	0.39 – 0.81	0.41	0.29 – 0.53
		10	4.11	2.56 – 5.67	7.35	4.34 – 10.36
		20	7.04	4.69 – 9.40	14.44	10.49 – 18.39
		30	9.78	6.88 – 12.68	19.89	15.25 – 24.53
		45	15.09	11.36 – 18.82	27.79	22.16 – 33.42
		60	29.86	23.69 – 36.02	33.80	27.24 – 40.35
Convalescent	**18**	0	0.72	0.28 - 1.16	0.40	0.15 – 0.65
		10	2.00	0.40 – 3.62	5.98	1.66 – 10.31
		20	3.21	0.81 – 5.61	10.84	3.29 – 18.38
		30	6.50	2.12 – 10.87	16.80	7.70 – 25.89
		45	11.26	5.24 – 17.28	23.39	13.13 – 33.66

	60	19.94	10.49 – 29.40	30.85	17.92 – 43.78

Intraphagosomal reporter bead association with neutrophils is significantly higher in whole blood stimulated with P4 peptide from patients admitted to critical care with severe sepsis.

Table demonstrates intraphagosomal reporter bead association with neutrophils after incubation for 0, 10, 20, 30, 45 and 60 minutes with P4 peptide (364µg/ml) and vehicle control (DEPC water) in three phases of infection (Early = day 0, Latent = day 7 and Convalescent = 3-6 months later). Neutrophils were selected gated on flow cytometry by forward and side scatter. Association with intraphagosomal reporter beads was determined by concomitant expression of the calibrator flour (pacific blue) of the intraphagosomal reporter beads. This raw data was used for the mixed model regression analysis described in Figure 2 of the main manuscript. Mean and 95% confidence intervals, statistical analysis by paired t-test.

Whole blood phagocytosis assay neutrophil-bead association: adjusted data

Variable	Coef.	Std. Err.	P value	95% C.I.
Latent phase (vs. early)	-7.58	0.81	<0.001	-9.17 – -5.99
Convalescent phase (vs. early)	-9.69	1.05	<0.001	-11.75 – -7.63
Incubation 10min (vs. 0 min)	4.38	1.58	0.006	1.28 – 7.47
Incubation 20min (vs. 0 min)	8.58	1.58	<0.001	5.49 – 11.68
Incubation 30min (vs. 0 min)	11.65	1.58	<0.001	8.56 – 14.74
Incubation 45min (vs. 0 min)	16.67	1.58	<0.001	13.58 – 19.77
Incubation 60min (vs. 0 min)	29.11	1.58	<0.001	26.02 – 32.20
xP4 (vs. control) at 0 min	-0.26	1.58	0.868	-3.35 – 2.83
Incubation 10min xP4 (vs. control)	3.91	2.23	0.080	-0.46 – 8.28
Incubation 20min xP4 (vs. control)	8.57	2.23	<0.001	4.20 – 12.94

Incubation 30min xP4 (vs. control)	12.38	2.23	<0.001	8.00 – 16.76
Incubation 45min xP4 (vs. control)	13.76	2.23	<0.001	9.39 – 18.13
Incubation 60min xP4 (vs. control)	7.84	2.23	<0.001	3.46 – 12.21
Constant	5.33	1.68	0.001	2.05 – 8.62

Random-effects parameters	**Estimate**	**Std. Err.**	**C.I.**
Individual variance	8.52	0.93	6.89 – 10.55
Residual variance	10.81	0.23	10.37 – 11.28

Mixed model regression analysis to demonstrate factors and interactions that influence intraphagosomal reporter bead association with neutrophils. The dependent outcome variable is intraphagosomal reporter bead association with neutrophils calculated as the proportion of neutrophils identified by flow cytometry that fluoresce with pacific blue (calibrator fluor of intraphagosomal reporter beads). The constant value represents the proportion of neutrophils associated with a bead(s) in study participants at incubation time 0 minutes when exposed to vehicle control (DEPC water). When whole blood and intraphagosomal beads were stimulated with P4 peptide (364μg/ml) there was significant interaction with the length of incubation (Incubation xP4). The proportion of neutrophils associated with an intraphagosomal reporter bead(s) was significantly increased in the presence of P4 peptide at 20, 30, 45 and 60-minute incubations but not at 0 or 10 minutes. This data was used in linear comparison analysis to construct data for Figure 2, main manuscript. Number of observations: 1127 (groups: 51). Coef = coefficient, C.I. = 95% confidence interval, Std. Err. = standard error.

Whole blood phagocytosis assay: neutrophil intraphagosomal reporter bead oxidation analysis

Raw, non-adjusted, data from the whole blood phagocytosis assay reporter bead oxidation output is presented below. This data was used to generate the mixed-model regression analysis to adjust for the hierarchical nature of sampling (one patient sampled three times).

The following table details the mixed-model regression analysis for intraphagosomal reporter bead oxidation, including the interaction between P4 peptide and incubation period. Coefficients from this analysis were used in linear comparison analyses to generate point estimates and 95% confidence intervals used to generate Figure 2B in the main manuscript.

Whole blood phagocytosis assay neutrophil intraphagosomal bead oxidation: raw data

Sample	n	Incubation	Control	C.I.	P4	C.I.
Early	**44**	0	0.92	0.79 – 1.05	0.87	0.75 - 1.00
		10	1.55	1.39 – 1.72	1.86	1.65 – 2.07
		20	1.72	1.51 – 1.92	2.10	1.85 – 2.35
		30	1.76	1.56 – 1.96	2.11	1.84 – 2.39
		45	1.81	1.57 – 2.06	2.10	1.83 – 2.37
		60	1.88	1.62 – 2.15	1.93	1.68 – 2.18
Latent	**32**	0	0.84	0.72 – 0.96	0.89	0.77 – 1.02
		10	1.33	1.17 – 1.49	1.74	1.45 – 2.03
		20	1.52	1.30 – 1.73	1.99	1.52 – 2.45
		30	1.55	1.40 – 1.70	1.91	1.70 – 2.12
		45	1.70	1.46 – 1.94	1.93	1.68 – 2.17
		60	1.84	1.56 – 2.13	1.92	1.65 – 2.19
Convalescent	**18**	0	0.66	0.53 – 0.79	0.78	0.62 – 0.94
		10	1.10	0.88 – 1.32	1.41	1.17 - 1.66
		20	1.28	0.98 – 1.59	1.76	1.27 – 2.26
		30	1.30	1.05 – 1.54	1.71	1.36 – 2.06
		45	1.40	1.13 – 1.68	1.96	1.51 – 2.41
		60	1.64	1.26 – 2.04	1.94	1.44 – 2.47

Intraphagosomal reporter bead oxidation ratio is significantly higher in whole blood stimulated with P4 peptide from patients admitted to critical care with severe sepsis. Table demonstrates the oxidation ratio of intraphagosomal reporter beads associated with neutrophils after incubation for 0, 10, 20, 30, 45 and 60 minutes with P4 peptide (364μg/ml) and vehicle control (DEPC water) in three phases of infection (Early = day 0, Latent = day 7 and Convalescent = 3-6 months later). Oxidation ratio is calculated: median fluorescence intensity FITC (reporter fluor) divided median fluorescence intensity pacific blue (calibrator fluor). This raw data was used for the mixed model regression analysis described in Figure 2 of the main manuscript. Mean and 95% confidence intervals, statistical analysis by paired t-test.

Whole blood phagocytosis assay neutrophil intraphagosomal bead oxidation: adjusted data

Variable	Coef.	Std. Err.	P value	95% C.I.
Latent phase (vs. early)	-0.26	0.04	<0.001	-0.34 – -0.19
Convalescent phase (vs. early)	-0.30	0.05	<0.001	-0.40 – -0.20
Incubation 10min (vs. 0 min)	0.55	0.08	<0.001	0.40 – 0.69
Incubation 20min (vs. 0 min)	0.72	0.08	<0.001	0.57 – 0.87
Incubation 30min (vs. 0 min)	0.76	0.08	<0.001	0.61 – 0.91
Incubation 45min (vs. 0 min)	0.86	0.08	<0.001	0.71 – 1.00
Incubation 60min (vs. 0 min)	0.98	0.08	<0.001	0.83 – 1.13
xP4 (vs. control) at 0 min	0.02	0.08	0.802	-0.13 – 0.17
Incubation 10min xP4 (vs. control)	0.32	0.11	0.003	0.11 – 0.53
Incubation 20min xP4 (vs. control)	0.42	0.11	<0.001	0.21 – 0.62
Incubation 30min xP4 (vs. control)	0.35	0.11	0.001	0.14 – 0.56

Incubation 45min xP4 (vs. control)	0.30	0.11	0.005	0.09 – 0.51
Incubation 60min xP4 (vs. control)	0.09	0.11	0.416	-0.13 – 0.30
Constant	0.98	0.09	<0.001	0.81 – 1.15

Random-effects parameters	**Estimate**	**Std. Err.**	**C.I.**
Individual variance	0.48	0.05	0.39 – 0.59
Residual variance	0.52	0.01	0.50 – 0.54

Mixed model regression analysis to determine which factors influence intraphagosomal reporter bead oxidation ratio. The dependent outcome variable is intraphagosomal reporter bead oxidation ratio in gated (FSC, SSC) neutrophils) calculated as the median fluorescence intensity (MFI) of FITC (reporter fluor) divided by MFI Pacific blue (calibrator fluor). The constant value represents the oxidation ratio of intraphagosomal reporter beads in study participants at incubation time 0 minutes when exposed to vehicle control (DEPC water). When whole blood and intraphagosomal beads were stimulated with P4 peptide (364μg/ml) there was significant interaction with the length of incubation (Incubation xP4). Oxidation of intraphagosomal reporter beads was significantly increased when stimulated with P4 peptide at 10, 20, 30 and 45 minute incubations but not at 0 and 60 minutes This data was used in linear comparison analysis to construct data for Figure 2, main manuscript. Number of observations: 1127 (groups: 51). Coef = coefficient, C.I. = 95% confidence interval, Std. Err. = standard error.

Whole blood phagocytosis assay: monocyte-bead association analysis

Raw, non-adjusted, data from the whole blood phagocytosis assay monocyte-bead association output is presented below. This data was used to generate the mixed-model regression analysis to adjust for the hierarchical nature of sampling (one patient sampled three times).

The following table details the mixed-model regression analysis for monocyte-bead association. . Coefficients from this analysis were used in linear comparison analyses to generate point estimates and 95% confidence intervals used to generate the following figure.

Whole blood phagocytosis assay monocyte-bead association: raw data (A)

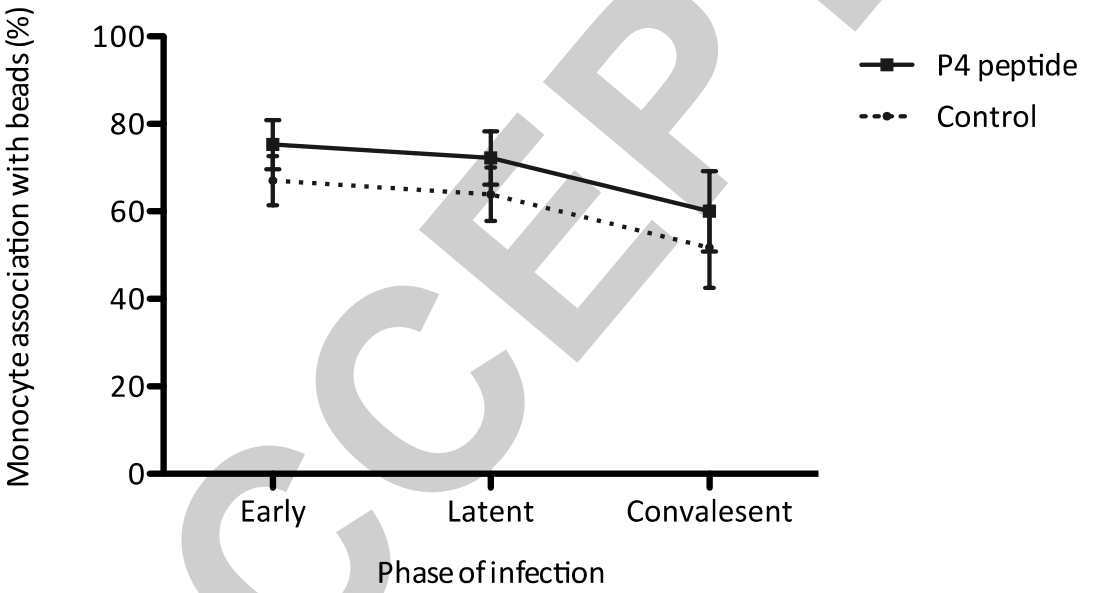
Sample	n	Control	IQR	P4	IQR	P value
Early	21	0.73	0.65 – 0.78	0.80	0.71 – 0.83	0.003
Latent	17	0.68	0.54 – 0.77	0.71	0.63 – 0.80	0.007
Convalescent	6	0.53	0.47 – 0.64	0.65	0.59 – 0.77	0.028

Whole blood phagocytosis assay monocyte-bead association: adjusted data (B)

Variable	Coef.	Std. Err.	Z	P value	C.I.
Latent phase (vs. early)	-0.03	0.03	-0.96	0.337	-0.09 – 0.032
Convalescent phase (vs. early)	-0.15	0.05	-3.25	0.001	-0.24 – -0.06
P4 peptide (vs. control)	0.08	0.03	3.30	0.001	0.03 – 0.13
Constant	0.67	0.03	23.20	<0.001	0.61 – 0.72

Random-effects parameters	Estimate	Std. Err.	C.I.
Individual variance	0.09	0.02	0.06 – 0.15
Residual variance	0.12	0.01	0.10 – 0.14

Monocyte association with intraphagosomal reporter beads. A: Table demonstrates median and interquartile range for the percentage of monocytes (identified via side scatter and CD14 expression by flow cytometry) that were associated with intraphagosomal reporter beads. Statistical analysis by Wilcoxon rank-sum test. **B:** Table demonstrates the mixed model regression analysis to identify independent predictors of monocyte-bead association (defined as above) adjusting for repeated measures from individual patients. The constant value represents the proportion of monocytes associated with intraphagosomal reporter beads when incubated with vehicle control for 45 minutes in early phase samples. When whole blood and intraphagosomal beads were stimulated with P4 peptide (364μg/ml) there was significant increase in association. There was a significant decrease in baseline (vehicle control) association in convalescent but not latent samples. Number of observations: 88 (groups: 31). Coef = coefficient, C.I. = 95% confidence interval, Std. Err. = standard error.



P4 peptide stimulates significantly increased monocyte association with intraphagosomal reporter beads in samples from patients admitted to critical care with severe infection. Figure displays the mixed-model adjusted proportion (point estimate and 95% confidence interval) of monocytes associated with an intraphagosomal reporter bead after 45 minutes incubation with whole blood. Monocytes were identified by flow cytometry (side scatter and CD14 expression) and association with bead was determined by co-expression of gated monocyte with bead calibrator

fluor. The addition of P4 peptide increased the mean difference in monocyte-bead association compared to vehicle (DEPC water) control in early (75.2% vs. 67.0%, p<0.001), latent (72.2% vs. 63.9%, p<0.001) and convalescent (60.0% vs. 51.7%, p<0.001) phases of infection.

Whole blood phagocytosis assay monocyte intraphagosomal reporter bead oxidation

Raw, non-adjusted, data from the whole blood phagocytosis assay monocyte intraphagosomal reporter bead oxidation is presented below. This data was used to generate the mixed-model regression analysis to adjust for the hierarchical nature of sampling (one patient sampled three times).

The following table details the mixed-model regression analysis for monocyte intraphagosomal reporter bead oxidation. In this analysis, P4 peptide stimulation was significantly associated with monocyte reporter bead oxidation but phase was not such that a figure has not been constructed with this data.

Whole blood phagocytosis assay monocyte intraphagosomal reporter bead oxidation: raw data (A)

Sample	n	Control	IQR	P4	IQR	P value
Early	21	0.91	0.67 – 1.35	1.08	0.96 – 1.69	<0.001
Latent	17	1.14	1.00 – 1.40	1.58	1.10 – 1.83	<0.001
Convalescent	6	1.00	0.82 – 1.05	1.32	1.08 – 1.62	0.028

Whole blood phagocytosis assay monocyte intraphagosomal reporter bead oxidation: adjusted data (B)

Variable	Coef.	Std. Err.	Z	P value	C.I.
P4 peptide	0.34	0.05	6.36	<0.001	0.23 – 0.44
Constant	1.05	0.08	14.02	<0.001	0.91 – 1.20

Random-effects parameters	Estimate	Std. Err.	C.I.
Individual variance	0.36	0.05	0.27 – 0.48
Residual variance	0.25	0.02	0.21 - 0.30

Oxidation ratio of intraphagosomal reporter beads associated with monocytes: A: Table demonstrates median and interquartile range for the oxidation ratio of intraphagosomal reporter beads that are associated with gated (SSC/CD14) monocytes (data not normally distributed). Oxidation ratio calculated as the median fluorescence intensity (MFI) of FITC (reporter fluor) divided by MFI Pacific blue (calibrator fluor). Statistical analysis by Wilcoxon rank-sum test. **B:** Table demonstrates the mixed model regression analysis to identify independent predictors of oxidation ratio of beads associated with monocytes (defined as above) adjusting for repeated measures from individual patients. The constant value represents the oxidation ratio when monocytes and beads were incubated with vehicle control for 45 minutes. When whole blood and intraphagosomal beads were stimulated with P4 peptide (364μg/ml) there was significant increase in oxidation. Phase of sepsis was not an independent predictor of oxidation in this analysis. Number of observations: 88 (groups: 31). Coef = coefficient, C.I. = 95% confidence interval, Std. Err. = standard error.

Whole blood phagocytosis assay positive control

Paired LPS positive control samples (n=105) at 45 minutes incubation demonstrated significantly increased bead association (median 27.1% vs. 13.3%, $p<0.001$) and oxidation ratio (median 1.57 vs. 1.41, $p<0.001$) compared to vehicle control samples but no significant difference in association (median 27.1% vs. 26.2%, $p=0.313$) and a significantly decreased oxidation ratio (median 1.57 vs. 1.73, $p=0.001$) compared to P4 peptide stimulated samples, Wilcoxon signed rank tests.

Figure S1: CONSORT diagram

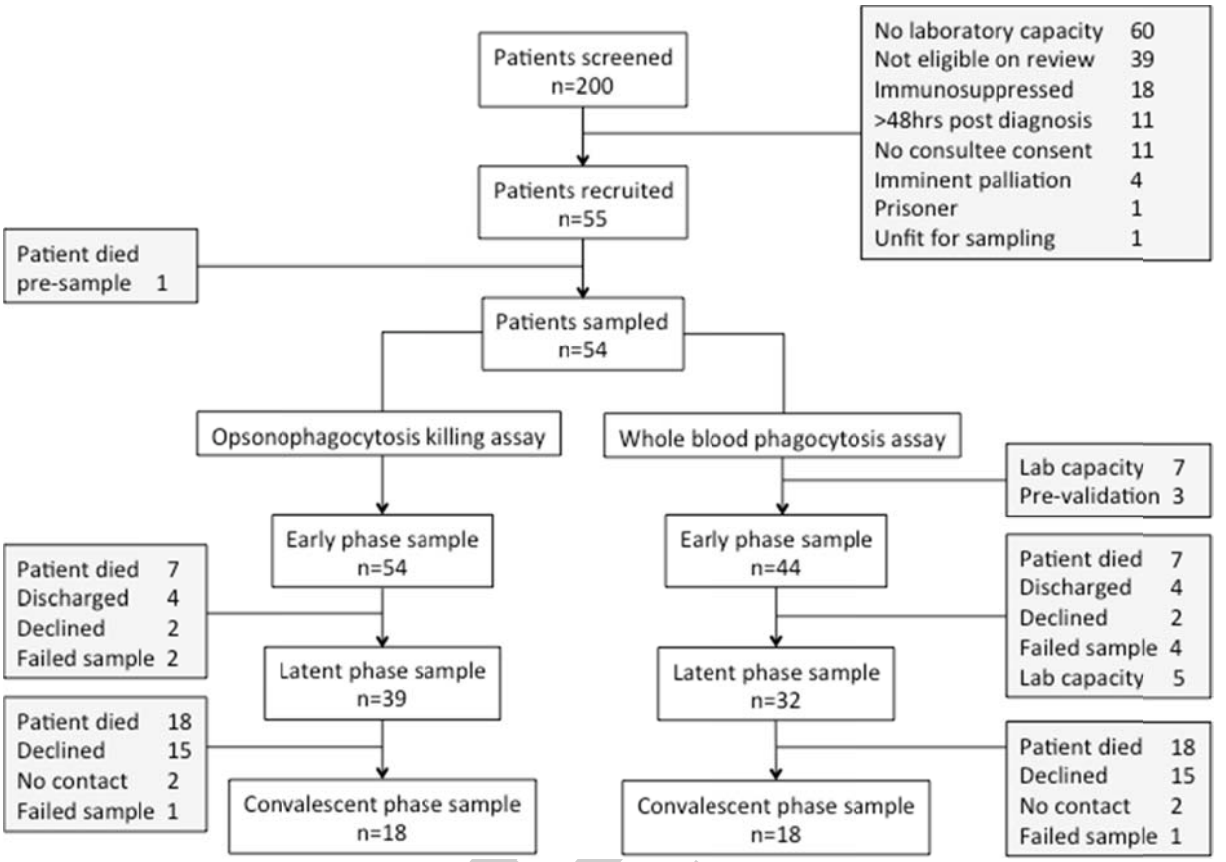
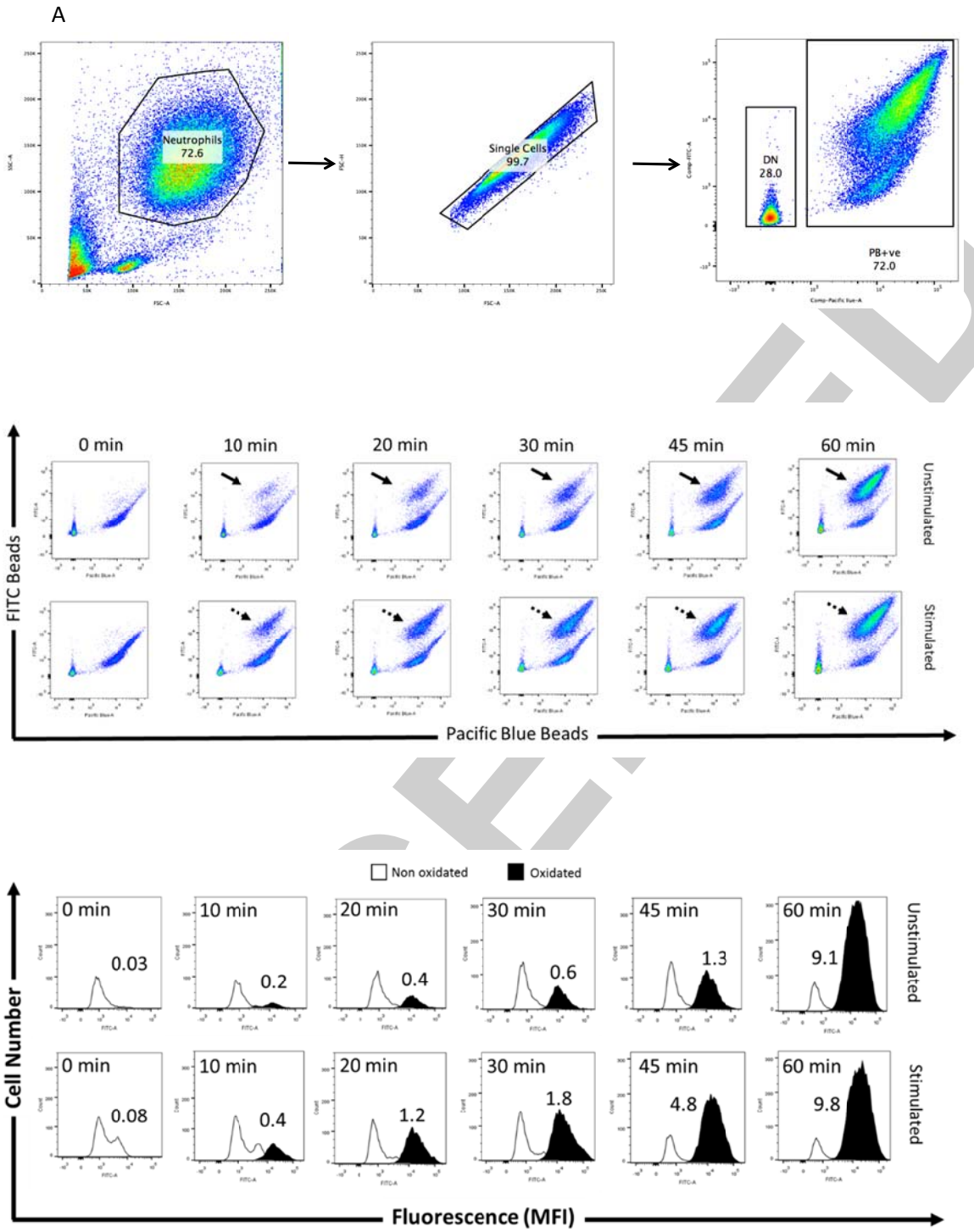


Figure S1: CONSORT diagram. Figure demonstrates patients recruited to the study period and the number of samples. Completed sample calculations relate to the total number of patients recruited to the study.

Figure S2: Flow cytometry gating strategies for the whole blood phagocytosis assay



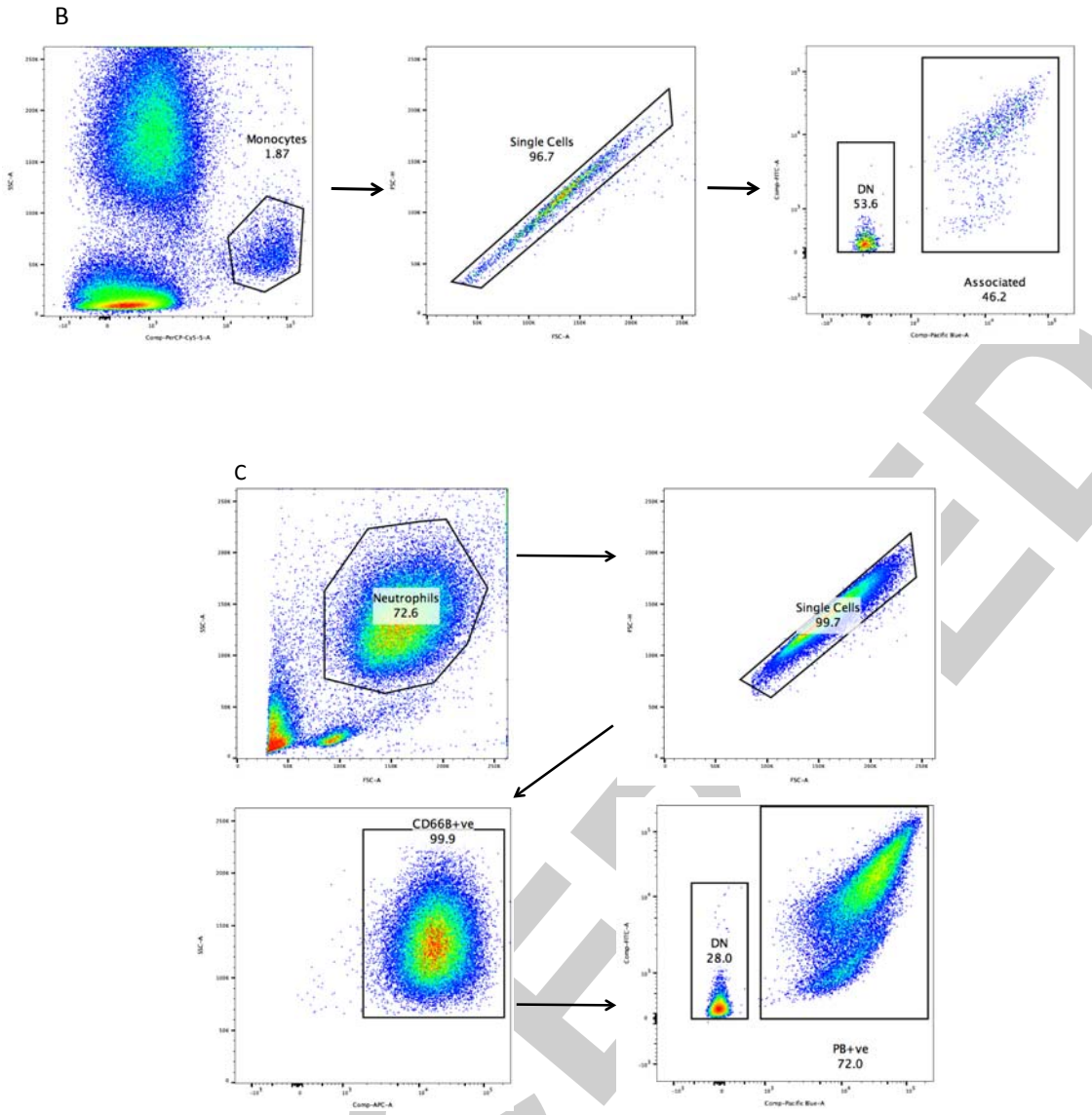


Figure S2: Flow cytometry gating strategies for the whole blood phagocytosis assay. A: Figure demonstrates the neutrophil gating strategy: forward (FSC-A) and side scatter (SSC-A) were used to identify and acquire 50,000 neutrophils per sample. Singlets were then identified and within this gate the expression of Intraphagosomal reporter bead Pacific Blue (calibrator fluor) and FITC (reporter fluor) were measured. **B:** Figure demonstrates the monocyte gating strategy. Intraphagosomal reporter beads were found to bleed into the FSC-A/SSC-A gate for monocytes so we used cell surface CD14 expression and SSC-A to identify the monocyte subpopulation. Singlets were then identified and within this gate expression of intraphagosomal bead reporter fluor and calibrator fluor were measured as above. **C:** Figure demonstrates neutrophil maturity gating strategy: FSC and SSC were used to identify and acquire 50,000 neutrophils per sample and singlets were identified as above. Cell surface CD66B was used as a neutrophil maturity marker (APC) with intraphagosomal reporter bead calibrator and reporter fluor subsequently measured within this population.

Figure S3: M peptide, an alternative control for P4 did not increase neutrophil bacterial killing

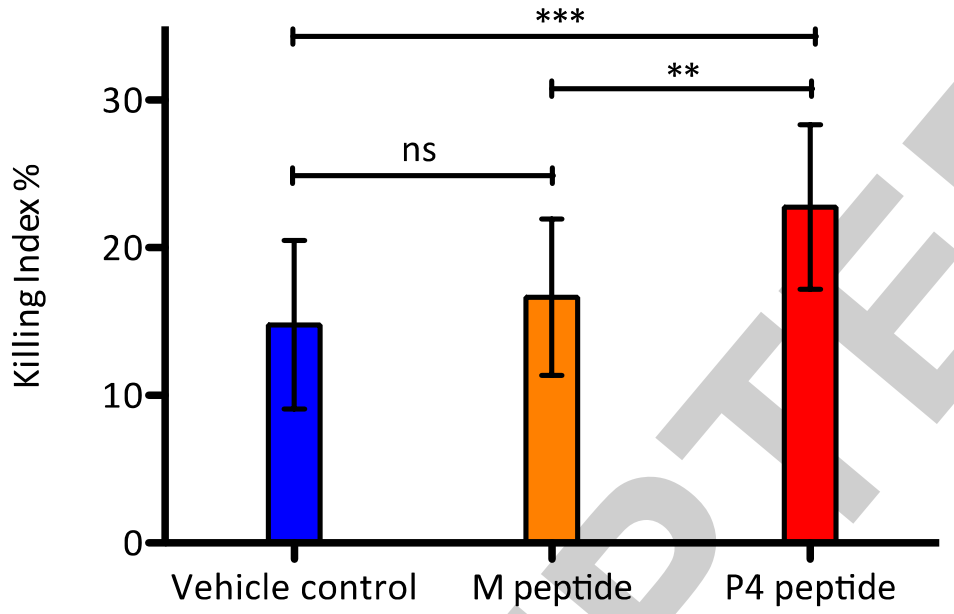


Figure S3: Control solutions for P4 peptide. Figure displays mean killing index and 95% confidence intervals for P4 peptide, M peptide and Vehicle (DEPC water) control (n=12). Mean bacterial killing was significantly higher for P4 peptide (22.77 C.I. 17.2-28.3) compared to M peptide (16.65 C.I. 11.4-21.9, p=0.003) and vehicle control (14.78 C.I. 9.1-20.5, p=0.001). There was no significant difference between M peptide and vehicle controls (p=0.366). Bacterial killing index was calculated as the proportion of colony forming units killed by neutrophils: (1 – (CFU count experimental well / CFU count non-neutrophil control well). The data were normally distributed (Shapiro-Wilk) and analysis performed by paired t-test.

Figure S4: Whole blood phagocytosis assay raw healthy volunteer data.

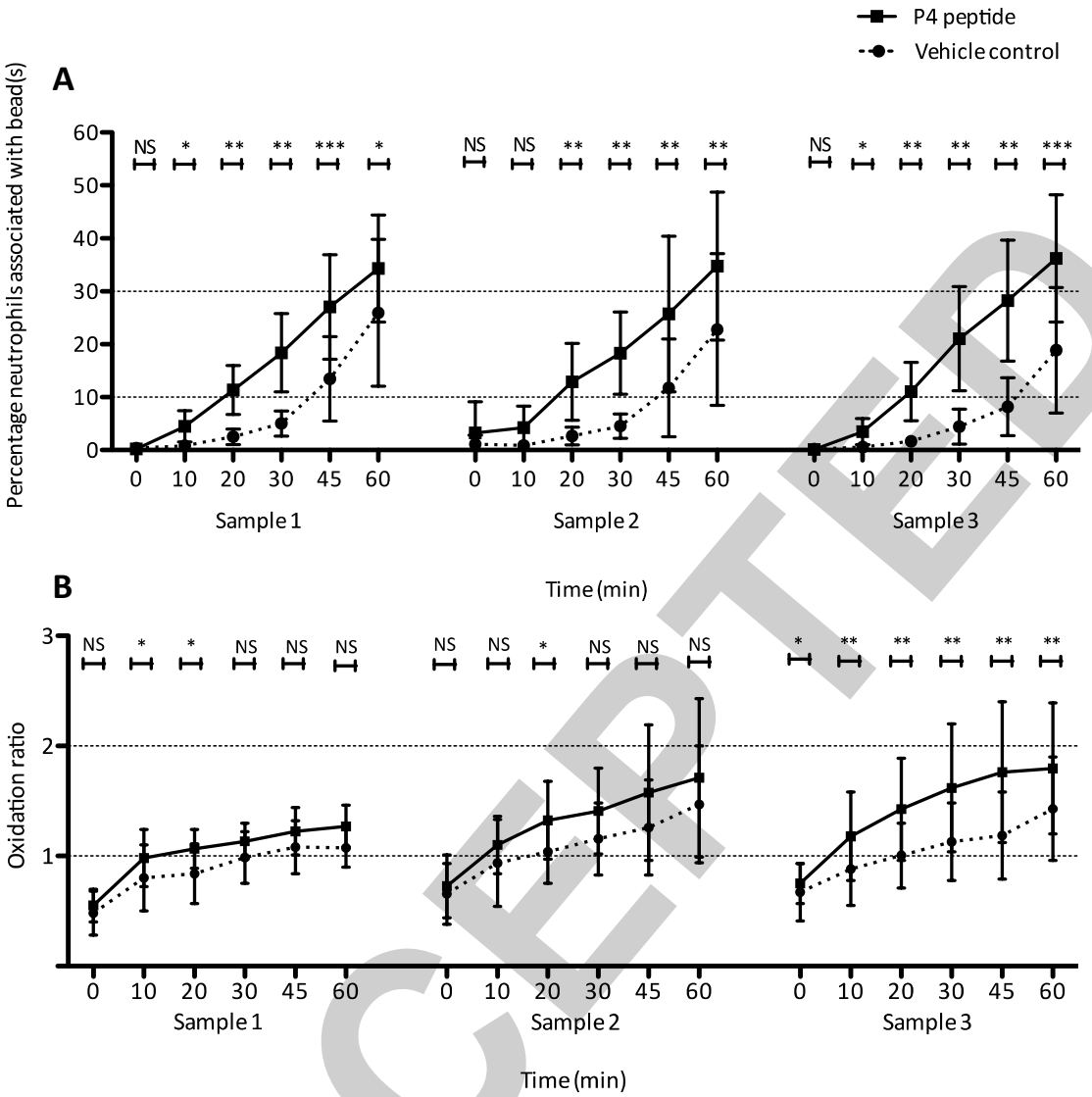


Figure S4: Healthy volunteer samples and the influence of P4 peptide. A: Figure demonstrates non-adjusted data for healthy volunteer neutrophil association with intraphagosomal reporter beads at 0, 10, 20, 30, 45 and 60 minutes in sample 1 (n=8), sample 2 (n=9) and sample 3 (n=11) with P4 peptide and vehicle control. **B:** Figure demonstrates non-adjusted data for healthy volunteer neutrophil oxidation of intraphagosomal reporter beads at 0, 10, 20, 30, 45 and 60 minutes in sample 1 (n=8), sample 2 (n=9) and sample 3 (n=11) with P4 peptide and control. Displayed are mean values and confidence intervals. Eleven volunteers were recruited in total for the study – laboratory capacity limited ability to process all blood samples at time points 1 and 2.

Figure S5: An illustration of area under the curve calculation for the whole blood phagocytosis assay, used to test association between potential clinical predictors and patient outcomes

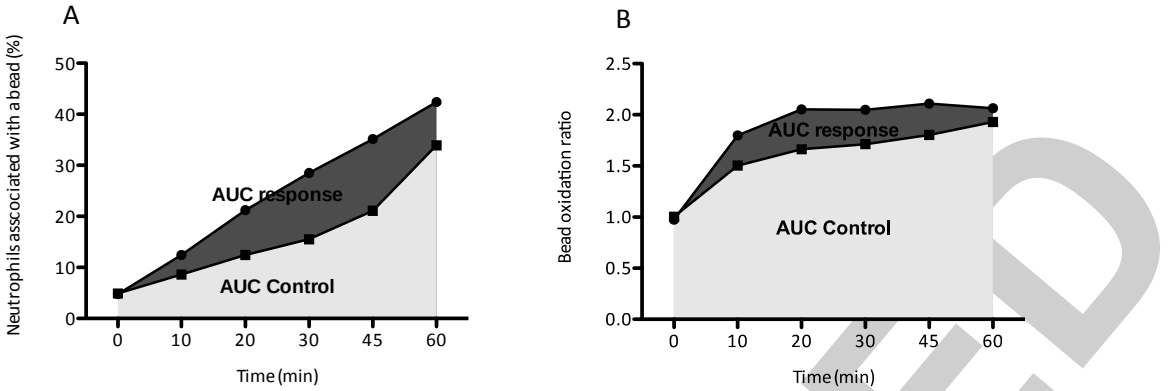


Figure S5: Whole blood phagocytosis area under the curve calculation illustration. A: Figure illustrates how the proportion of neutrophils (%) associated with intraphagosomal bead(s) changes according the length of incubation. An area under the curve (AUC) was calculated for the vehicle control samples (light grey shaded area) and for the absolute response to P4 peptide (dark grey shaded area): AUC P4 peptide response minus AUC vehicle control. **B:** Figure illustrates how the oxidation ratio of intraphagosomal repoter beads associated with neutrophils changes according the length of incubation. An AUC was calculated for the vehicle control samples (light grey shaded area) and for the absolute response to P4 peptide (dark grey shaded area): AUC P4 peptide response minus AUC vehicle control.

Bibliography

1. Levy MM, Fink MP, Marshall JC, et al. (2003) 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. Crit Care Med 31:1250–6. doi: 10.1097/01.CCM.0000050454.01978.3B

2. Levy MM, Dellinger RP, Townsend SR, et al. (2010) The Surviving Sepsis Campaign: results of an international guideline-based performance improvement program targeting severe sepsis. Crit Care Med 38:367–74. doi: 10.1097/CCM.0b013e3181cb0cdc

3. McAuley DF, Laffey JG, O'Kane CM, et al. (2014) Simvastatin in the Acute Respiratory Distress Syndrome. N Engl J Med. doi: 10.1056/NEJMoa1403285

4. Barany G, Merrifield RB (1979) A chromatographic method for the quantitative analysis of the deprotection of dithiasuccinoyl (Dts) amino acids. Anal Biochem 95:160–70.

5. Reed RC, Louis-Wileman V, Cosmai E V, et al. (1997) Multiple antigen constructs (MACs): induction of sterile immunity against sporozoite stage of rodent malaria parasites, Plasmodium berghei and Plasmodium yoelii. Vaccine 15:482–8.

6. Verheul AF, Udhayakumar V, Jue DL, et al. (1995) Monopalmitic acid-peptide conjugates induce cytotoxic T cell responses against malarial epitopes: importance of spacer amino acids. J Immunol Methods 182:219–26.

7. Metzgar D, Zampolli A The M protein of group A Streptococcus is a key virulence factor and a clinically relevant strain identification marker. Virulence 2:402–12. doi: 10.4161/viru.2.5.16342

8. Bruner M, James A, Beall B, et al. (2003) Evaluation of synthetic, M type-specific peptides as antigens in a multivalent group A streptococcal vaccine. Vaccine 21:2698–703.

9. Romero-Steiner S, Frasch CE, Carlone G, et al. (2006) Use of opsonophagocytosis for serological evaluation of pneumococcal vaccines. Clin Vaccine Immunol 13:165–9. doi: 10.1128/CVI.13.2.165-169.2006

10. Miles AA, Misra SS, Irwin JO (1938) The estimation of the bactericidal power of the blood. J

Hyg (Lond) 38:732–49.

11. VanderVen BC, Yates RM, Russell DG (2009) Intraphagosomal measurement of the magnitude and duration of the oxidative burst. Traffic 10:372–8. doi: 10.1111/j.1600-0854.2009.00877.x