P4 therapy for the treatment of severe bacterial infections

Thesis submitted for the degree of Doctor of Philosophy

at The University of Liverpool by Suzanna Gore

July 2016

Declaration

This thesis is the result of my own work and effort. The material contained in the thesis has not been presented, nor is currently being presented, either wholly or in part, for any other degree or other qualification.

Research in this thesis was carried out at the Institute of Infection and Global Health (University of Liverpool, UK) and the Liverpool School of Tropical Medicine (Liverpool, UK).

Abstract

Background:
Severe bacterial infections and sepsis are a major global cause of mortality and morbidity and with the number of antibiotic resistance cases on the rise. Despite the introduction of treatment guidelines such as those implemented by the surviving sepsis campaign mortality remains high and new therapies are desperately needed. One new therapy, which may be of benefit, is P4 therapy – the combination of the immunomodulating peptide P4 and intravenous immunoglobulin (IVIG). The ability of P4 to augment phagocytosis in models of pneumococcal infections, decreasing bacterial burden and improving survival has previously been shown. This thesis goes on to investigate the efficacy of P4 therapy in models of Gram-negative infections, with and without antibiotics and in *ex vivo* studies from patients with severe community acquired pneumonia (CAP).

Methods:
Murine models of *Escherichia coli* and *Klebsiella pneumoniae* infection were used to evaluate the efficacy of P4 peptide with IVIG in the treatment of severe Gram-negative infections. Flow cytometry and ELISA were used to assessed immune responses to infection with P4 treatment.
Neutrophils from patients with severe CAP were isolated and their responses to P4 assessed with *ex vivo* phagocytosis assays and flow cytometry. *In vivo* and *ex vivo* studies were performed with naïve mice and tissue culture cell lines to evaluate the effect of P4 on neutrophil receptor expression and the binding of P4 peptide to cells.

Results:
Treatment with P4 and IVIG in combination with antibiotics led to significant improvements in survival and bacterial burden in *Klebsiella pneumoniae* infection. Treatment of *Escherichia.coli* infection with P4 and IVIG in combination with antibiotics showed no benefits over treatment with antibiotic with IVIG, this was likely due to the infection being too severe.
In neutrophils from CAP patients increases in bacterial killing when treated with P4 in phagocytosis assays were seen in 60% of patient. Patients who did not respond to the P4 treatment showed higher levels of IL-8 and IL-10 in their serum and higher disease severity scores.

Conclusions:
P4 treatment showed efficacy in the treatment of *Klebsiella* infection but data from *E. coli* infections and *ex vivo* treatment of CAP patient neutrophils suggest that infection severity and levels of IL-8 and IL-10 may effect treatment success. P4 could be a potential new treatment option for patients with severe bacterial infections but further studies are needed to better establish which patients would benefit from this treatment and the influence of host immune status on treatment efficacy.

Acknowledgements

This thesis would not have been possible without the support of my supervisors Aras Kadioglu and Stephen Gordon, whose guidance and encouragement has seen me through the past five years and I will always be grateful for. I'd also like to thank our collaborators Edwin Ades and Gowrisankar Rajam for their support and enthusiasm throughout this project.

Thanks to the members of our research group past and present and fellow scientists who have helped me along the way, especially Daniel Neill, Elaine Waters, Mathieu Bangert, Stavros Panagiotou, Simon Abrams and Toni Banyard, who have given me so much support, patience and friendship over the years.

I am thankful to the friends I have made during my time at the institute who have been there when I needed someone to commiserate with, complain to or just when what I really needed was a drink – Thanks to Simon, Stacey, Matt, Dan, Jen, Yasmina, Laura and Steph.

Finally, I'd like to thank my parents Helen and Jeremy and my family: James, Robert, Olive, Danielle and Gordon. I'm grateful for the love, support and encouragement.

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List of abbreviations:

APACHE II	Acute Physiology and Chronic Health Evaluation II
BAL	Bronchoalveolar lavage
BHI	Brain heart infusion
CAP	Community acquired pneumonia
CFU	Colony forming unit
CNS	Central nervous system
COPD	Chronic pulmonary obstructive disease
DEPC	Diethylpyrocarbonate treated water
EOS	Early onset sepsis
FBS	Fetal bovine serum
FcγR	Fc gamma receptor
GBS	Group B *Streptococcus*
GI	Gastrointestinal
ICU	Intensive care unit
Ig	Immunoglobulin
INF	Interferon
IL	Interleukin
IP	Intraperitoneal
IQR	Interquartile range
IVIG	Intravenous immunoglobulin
IV	Intravenous
LOS	Late-onset sepsis
LRTI	Lower respiratory tract infection
LPS	Lipopolysaccharide
MPO	Myeloperoxidases
NET	Neutrophil extracellular trap
OPK	Opsonophagocytosis killing assay
P4	Immunoactivating peptide
PBS	Phosphate buffered saline
PsaA	Pneumococcal surface adhesin A
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SEM	Standard error of the mean
SOFA	Sequential organ failure assessment
UTI	Urinary tract infection

Chapter I. Introduction

A. Sepsis

The word sepsis dates back over 2000 years to ancient Greece, when Hippocrates defined sepsis, σήψις, as a process similar to putrefaction, the festering of wounds (1). Since that date sepsis has varied in meaning as understanding of the condition has developed, arriving at its current clinical definition as: a life-threatening organ dysfunction caused by a dysregulated host response to infection and septic shock defined as sepsis with persisting hypotension despite adequate volume resuscitation (2).

1. Disease burden

Sepsis presents a significant burden on healthcare systems; global annual cases are documented at 1.8 million although due to low rates of recognition this is likely an underestimate (3). In England alone there were 122,822 cases between 2013 and 2014 and the number of cases is rising at a rate of around 10% year on year, this is likely due to people living longer with more chronic illnesses (4). Case-fatality rates for sepsis vary depending on severity: sepsis has a mortality of 30%, whereas severe sepsis and septic shock have mortality rates of 50 and 80% respectively (5). Mortality and incidence of sepsis also vary greatly with age; below 40 years in hospital mortality is between less than 5% and 15% whereas in those above 40 years there is a linear relationship between age and mortality. Incidence is highest in those under five years, is low between five and 40 years and then increases with age (6). Severe infections can progress into sepsis due to factors such as treatment failure and patient immune status; the most

common source of infection in sepsis is the respiratory tract followed by the genitourinary and gastrointestinal tracts (7).

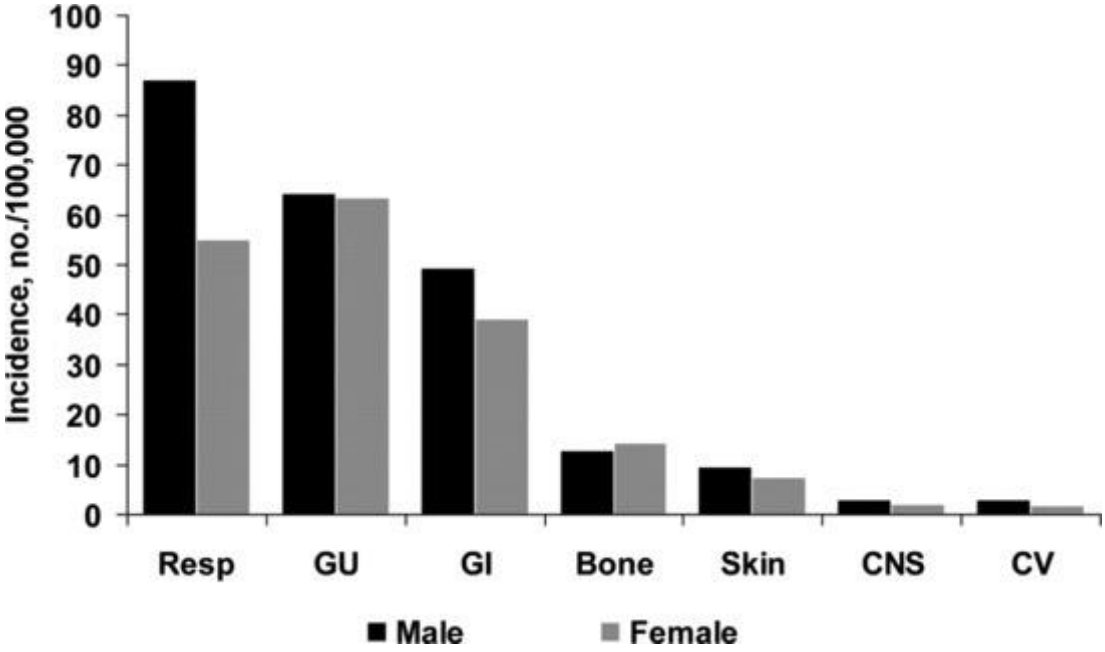


Figure 1. Incidence of sepsis according to source of infection, stratified by sex. From Moss et al. 2005 (7)

2. Diagnosis

Until this year (2016) sepsis diagnosis followed fairly rigid criteria: evidence of suspected infection and 2 or more SIRS criteria:

- Temperature >38°C or <36°C
- Heart rate >90/min
- Respiratory rate >20/min or $PaCO_2$ <32 mm Hg (4.3 kPa)
- White blood cell count >12 000/mm^3 or <4000/mm^3 or >10% immature bands

This definition, which focused in on excess inflammation was deemed to be unhelpful in diagnosis as it had poor discriminant and concurrent validity

when identifying potential sepsis patients and fails to take into account the role of anti-inflammatory processes in disease progression (2).

The new guidelines for the identifying of sepsis patients (workflow detailed in figure 2) are intended to ease early diagnosis of a condition which is protean in nature and in which a myriad of clinical signs and symptoms can be used for identification, many of which can be influenced by both pathogen and host heterogeneity. The quick SOFA (qSOFA) score was introduced to help highlight patients that were likely to have sepsis and are in need of further investigation (2).

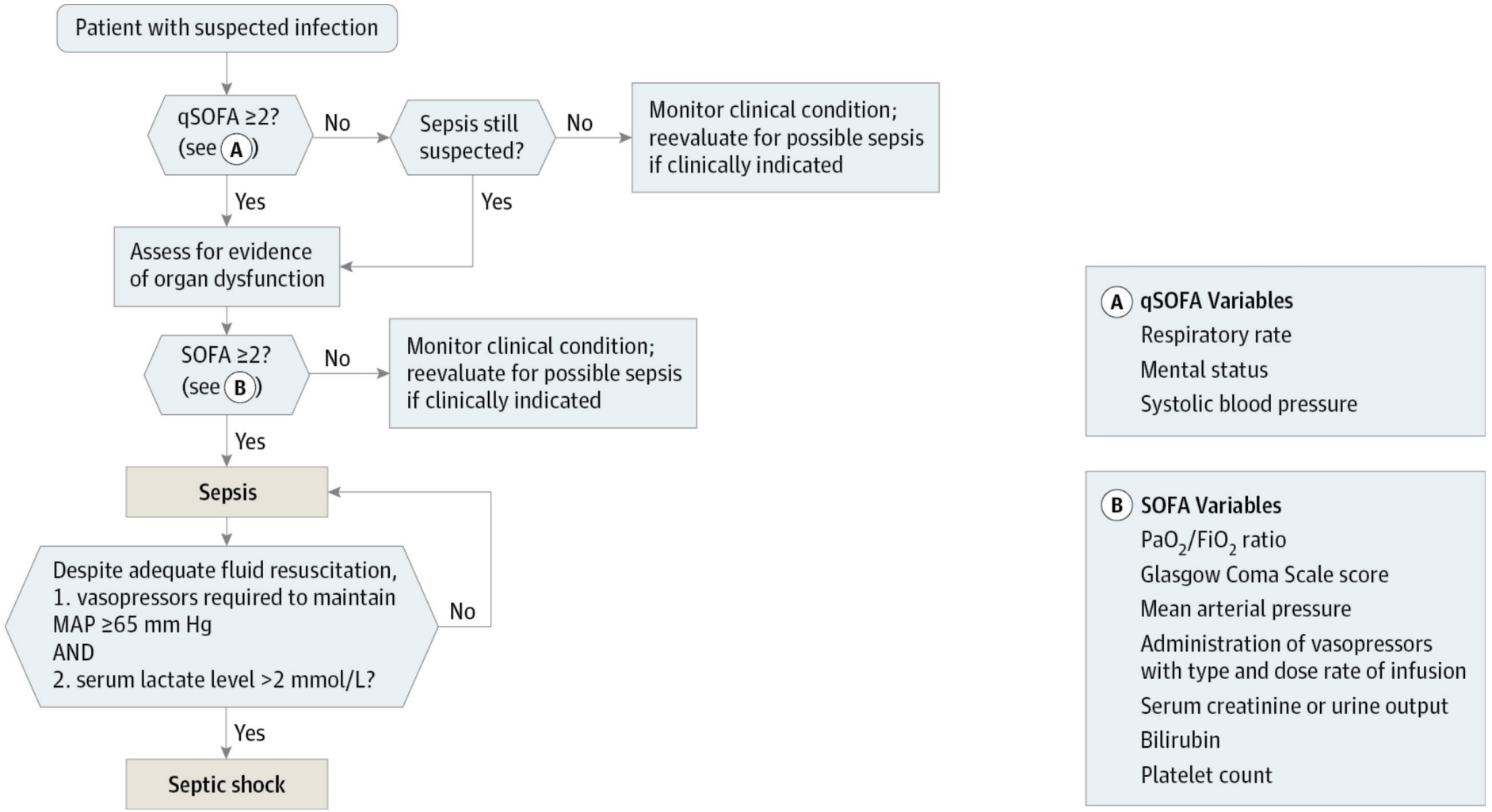


Figure 2 Sepsis diagnosis – From Singer *et al* 2016

3. Costs of sepsis

Sepsis is associated with high costs, both to healthcare systems and to the patients themselves. Sepsis patients account for almost one third of ICU admissions in the UK and almost half of these patients die in hospital (8). The cost of a single day in ICU in a European hospital ranges from €1168 - €2025 with ICU departments estimated to consume 20% of the total hospital budget (9). Figure 3 shows the breakdown of these direct costs, with the biggest expensive being staffing.

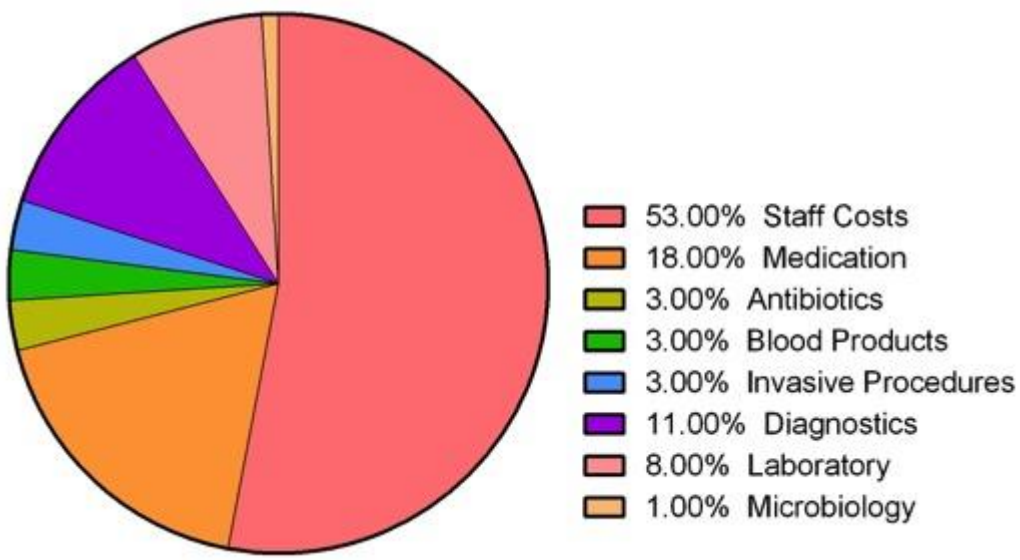


Figure 3 Direct costs of sepsis in German ICUs
Direct costs in German intensive care units (ICUs). Medication = drugs, fluids, nutrition. Invasive procedures includes diagnostic procedures, renal replacement therapy, and mechanical ventilation. Adapted from (10).

With sepsis patients making up such a high proportion of ICU patients they present a significant financial burden and are estimated to cost the NHS £2 billion a year. Direct cost of treatment per case of severe sepsis range from €23,000 to €29,000 in Europe and €34,000 in the US; this cost only represents 20-30 % of the total cost with 70-80 % of costs being indirect, mainly due to productivity losses (11).

As well as high fiscal costs patients can also suffer from long term sequela such as cognitive and physical impairments, muscle weakness, wasting and fatigue as well as the worsening of existing chronic conditions (12-14). Sepsis patients are 1.5 times more likely to be readmitted to hospital within 30 days of discharge than non-sepsis patients and are also more likely to die or move into hospice care (15).

B. Gram negative infections

Although Gram-positive infections are the most common cause of sepsis, having superseded Gram-negative infections as the leading cause in the mid-late 1980s, Gram-negative infections still constitute a high proportion of cases (16). Gram-negative infections account for only 38% of reported cases sepsis (Gram-positive cases constitute 52% of cases, while fungal infections constitute 10%) but they are highly prevalent as bloodstream infections and infections with *Escherichia coli* and *Klebsiella spp.* are on the rise (17).

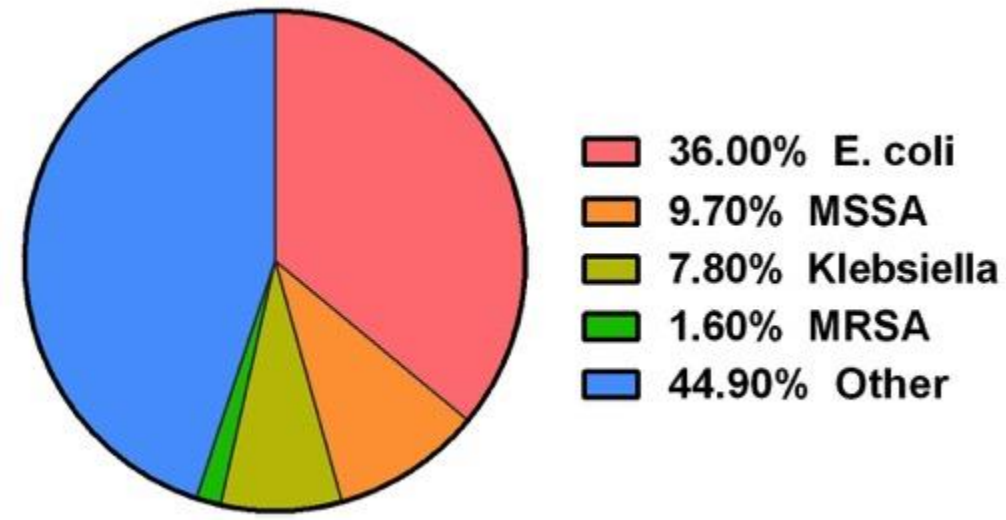


Figure 4 Blood infection-causing organisms
In adults England, Wales and Northern Ireland April 2011 – May 2012. Source: Department of Health

1. *Escherichia coli*

E. coli is an extremely versatile microorganism and is the most prevalent commensal in the gut of humans and warm-blooded animals. As well as being a harmless commensal, *E. coli* also exists as a number of pathotypes capable of causing disease in health humans. Of these pathotypes (listed in Table 1) the extra-intestinal *E.coli:* uropathogenic (UPEC) and neonatal

meningitis (NMEC) are of particular interest as the causative agents of urosepsis and neonatal meningitis and sepsis.

Table 1 E. coli pathotypes

Pathotype (acronym)	Diseases
Enteric E.coli	
EnteroPathogenic *E. coli* (EPEC)	Diarrhoea in children
EnteroHaemorrhagic *E. coli*(EHEC)	Haemorrhagic colitis, haemolytic-uremic syndrome
EnteroToxigenic *E. coli* (ETEC)	Traveller's diarrhoea
EnteroAggregative *E. coli* (EAEC)	Diarrhoea in children
Diffusely Adherent *E. coli*(DAEC)	Acute diarrhoea in children
EnteroInvasive *E. coli* (EIEC)	Shigellosis-like
Adherent Invasive *E. coli* (AIEC)	Associated with Crohn disease
Extraintestinal E. coli (ExPEC)	
UroPathogenic *E. coli* (UPEC)	**Lower UTI and systemic infections**
Neonatal Meningitis *E. coli* (NMEC)	**Neonatal meningitis**
Avian Pathogenic *E. coli* (APEC)	Probable source of food-borne disease

From *Allocati et al*. 2013 (18)

a) Urosepsis

Urinary tract infections (UTI) are extremely common with approximately 150 million cases globally per year and they are the second most common reason for antibiotic prescription (19). In elderly patients, diabetics and immunosuppressed patients there is a higher risk of UTIs developing into urosepsis, this risk is multiplied further by hospitalisation where interventions

such as the placement of indwelling urethral catheter, ureteric stents and nephrostomy tubes further increase risk (20). Catheter-associated UTIs account for 40% of hospital acquired infections (19). *E. coli* is the most common cause of UTI and UPEC isolates account for 75-95% of total cases and 65% of hospital acquired UTIs (*Klebsiella spp, Pseudomonas aeruginosa* and *Proteus spp* have higher incidence in a hospital setting than the community) (21). Urosepsis constitutes 5% of sepsis cases overall with *E. coli* the causative agent in 50% of these cases (21). Urosepsis is becoming an increasing public health issues with the aging population and increases in antibiotic resistance, which is highly prevalent in UPEC strains due to their propensity to cause occult chronic infections.

b) Neonatal sepsis and meningitis

Infection remains a major cause of mortality in neonates with 7% of all deaths in children under five year old caused by neonatal sepsis (Figure 5). Neonatal sepsis can be divided into two groups: early-onset sepsis (EOS), within one week of birth (some studies state 72 hours) and late-onset sepsis (LOS), after one week from birth. EOS is thought to be due to maternal intrapartum transmission of infectious organisms whilst LOS is thought to be due to postnatal infection with preterm infants being particularly at risk as a result of prolonged hospitalization and use of indwelling catheters, endotracheal tubes, and other invasive procedures (22).

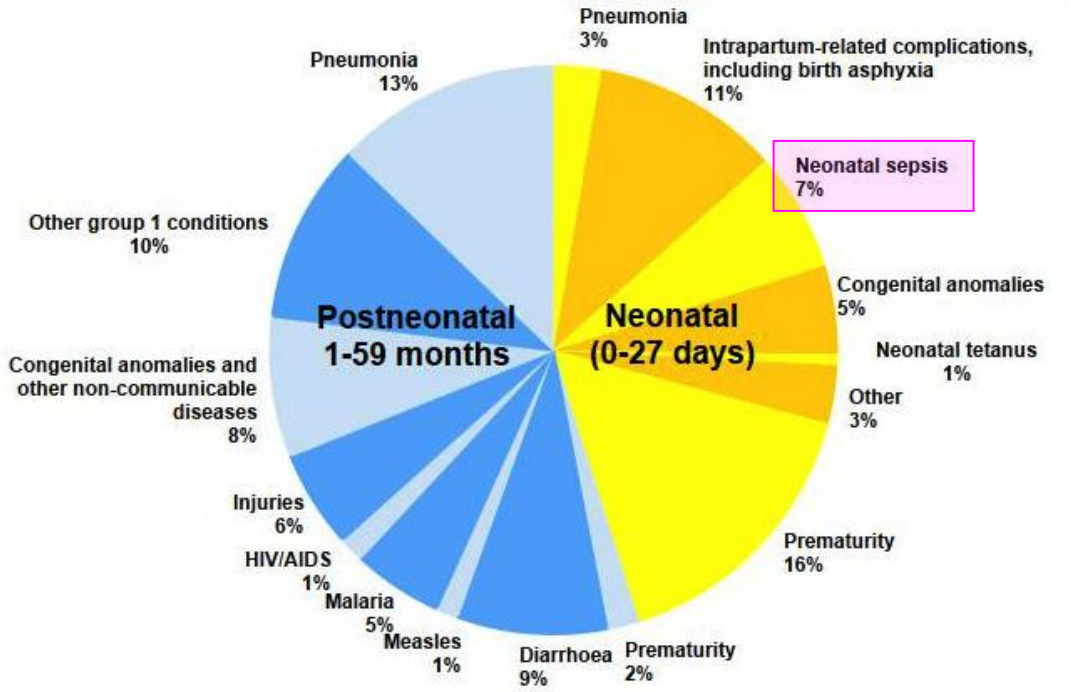


Figure 5. Cause of death in children under 5 years, 2015
Adapted from WHO 2016 (23)

Neonatal sepsis due to *E. coli* has increased in recent years and is the most common cause of early on-set sepsis in very low birth weight neonates (VLBW); those weighing less than 1500 grams. *E. coli* is often associated with more severe infections and meningitis and it has become the principal cause of sepsis-related mortality among VLBW infants (24.5%) and the second most common cause in infants at term (22).

Premature births are more susceptible to infection due to their immature immune systems; compared with adults, neonate cells have lower pro-inflammatory cytokine production, lower expression of neutrophil adhesion molecules, decreased response to chemoattractants and increased induction of IL-10 (24-26). The majority of transplacental passage of IgG occurs in the later stages of pregnancy with IgG concentrations only reaching 50% between weeks 28-32 of gestation, which means premature births have a reduced level of humoral immunity compared with term births (27).

Complement levels increase with age but are still significantly lower than adult levels at term, with a marked deficiency in the levels of C9 limiting the formation of the membrane attack complex (28).

Neonatal meningitis is primarily caused by *E. coli* isolates belonging to the NMEC pathotype, the condition has a high mortality rate of between 15 and 40% and is associated with severe long term neurological defects in survivors (29). Bacteraemia is a prerequisite to meningeal infection with the infection being spread haematogenously; 80% of NMEC isolates possess a K1 capsule, which although not necessary for traversing the blood-brain barrier is essential for survival (29, 30). Invasion of the blood-brain barrier by *E. coli* is a function of bacterial burden with the risk of invasion increasing with bacterial counts of more than 10^3 per ml of blood (31).

Treatment of neonatal infections are complicated as the drug dosing schedules and concentrations are based on the pharmacokinetics and pharmacodynamics data from adult trials. Intrapartum interventions for the prevention of Group B *Streptococcus* (GBS) infection (antibiotics for prophylaxis or suspected chorioamnionitis) have shown great benefits in reducing the incidence of EOS caused by GBS – no such intervention exists of *E. coli* and prevention remains a challenge (32). Improvements in the recognition of EOS are needed in order to help prevent the unnecessary treatment of neonates with antibiotics (33).

c) Peritonitis and intra-abdominal sepsis

The term intra-abdominal infection covers an array of different etiologies (see Table 2) and abdominal infection is the third most common cause of sepsis after pulmonary and genitourinary infection (34).

Table 2 Potential etiology of intra-abdominal infections (35)

Gastrointestinal	Anastomotic leak	Iatrogenic perforations
	Appendicitis	Inflammatory bowel disease
	Clostridium difficile colitis	Meckel diverticulum
	Diverticulitis	Peptic ulcer disease
	Fistula formation	Perforated neoplasm
	Gastrointestinal malignancy	Perforating trauma
Biliary	Acalculous cholecystitis	Ascending cholangitis
	Acute calculous cholecystitis	Intrahepatic abscess
Retroperitoneal	Acute pancreatitis	
	Kidney abscess	
	Pyelonephritis	
Pelvic	Endometritis	Parametritis
	Extrauterine pregnancy	Pelvic inflammatory disease
	Oophoritis	Salpingitis
	Ovarial abscess	Tubal abscess
Others	Blunt trauma	Spontaneous bacterial peritonitis
	Intrasplenic abscess	

Peritonitis can be categorised into primary, secondary or tertiary peritonitis. Primary peritonitis is defined as peritonitis with no obvious cause (such as breach of the GI tract), is usually monobacterial and its most frequent presentation is spontaneous bacterial peritonitis in patients with cirrhosis or ascites (35). Secondary peritonitis is caused by perforations of a hollow viscus due to inflammation or malignancy, less common causes include superinfection of ischemic necrosis, fistulas, trauma, or iatrogenic causes such as leakage from anastomotic sites (35). Tertiary peritonitis is defined as a persistent or recurrent peritoneal infection, usually the result of failed

treatment of a secondary peritonitis, 20% of secondary peritonitis patients progress to tertiary peritonitis and it is associated with a high incidence of nosocomial and multidrug resistant infections, high mortality, immune dysfunction and more severe organ dysfunction (35). Enterobacteriaceae are by far the most commonly isolated pathogens from peritoneal infections within which *E. coli* and *K. pneumoniae* predominate, although infections from secondary peritonitis are often polymicrobial (seeTable 3).

Table 3 Aerobic bacteria identified from intra-operative peritoneal fluid (36)

Total	1330 (100%)
Aerobic Gram-negative bacteria	957 (71.9%)
E. coli	548 (41.2%)
(*E. coli* resistant to third generation cephalosporins)	75 (5.6%)
K. pneuumoniae	140 (10.5%)
(*K. pneumoniae* resistant to third generation cephalosporins)	26 (1.4%)
K. oxytoca	11 (0.8%)
(*Klebsiella oxytoca* resistant to third generation cephalosporins)	2 (0.1)
Enterobacter	64 (4.8%)
Proteus	47 (3.5%)
Pseudomonas	74 (5.6%)
Others	73 (5.6%)
Aerobic Gram-positive bacteria	373 (29.1%)
Enterococcus faecalis	153 (11.5%)
Enterococcus faecium	58 (4.4%)
Staphylococcus Aureus	38 (2.8%)
Streptococcus spp.	85 (6,4%)
Others	39 (2.9%)

Patients older than 18 years undergoing surgery or interventional drainage to address

2. *Klebsiella pneumoniae*

Klebsiella spp. are ubiquitous in the environment as well as being a common commensal in the human and animal gastrointestinal tract. *K pneumoniae* and *K oxytoca* are the two species responsible for most human infections.

a) *K. pneumoniae in community acquired pneumonia*

Although thought to be an important cause of community acquired pneumonia between the 1920s-1960s *K. pneumoniae* is now a relatively uncommon cause of CAP with incidence in the US estimated at less than 1%. It should be noted however that diagnosis rates in CAP are low with failure to identify the etiological agent being as high as 65% (37, 38). In some regions (Taiwan and South Africa) and in specific patient groups (alcoholics and males with leukopenia) *K. pneumoniae* does still cause a significant proportion of CAP (39). These cases of primary CAP, caused by hypervirulent *K. pneumoniae* isolates (discussed further below) have higher mortality rates (55.1%) and marginally higher incidence (31%) than CAP caused by *S. pneumoniae* (mortality 27.3%) in these regions (39, 40).

K. pneumoniae in western countries is however still an important cause of nosocomial infections accounting for around 10% of hospital-acquired and ventilator-acquired bacterial pneumonias as well as being a common cause of hospital-acquired UTI and is also commonly found in the lungs of chronic pulmonary obstructive disorder (COPD) patients (38, 39, 41).

b) Hypervirulent Klebsiella pneumoniae

In addition to *K. pneumoniae* causing respiratory infections and UTIs, invasive liver abscess syndrome (defined as *K. pneumoniae* liver abscess with extrahepatic complications, particularly CNS involvement, necrotising fasciitis, or endophthalmitis) has become a significant problem, spreading out from Southeast Asia over the last 20 years and being increasingly identified in the west (42, 43). This infectious syndrome is of particular concern as it is caused by hypervirulent (also referred to as hypermucoviscous) isolates of *K. pneumoniae* capable of causing infection in healthy adults in the community and has mortality ranging from 3-42% despite infecting a relatively healthy group of patients compared with those infected with the "classical" *K. pneumoniae* isolates common in respiratory infections (44).

The reason for the increased virulence of these isolates is not well defined although higher production of siderophores (resulting in improved iron acquisition) compared to "classical" isolates is thought to play an important role (45). The hypermucoviscous phenotype is also thought to be significant factor, with the trait being used to provisionally identify hypervirulent isolates via a "string test" (Figure 6). Whether this phenotype is a result of increased capsule production or due to the presence of extracapsular polysaccharide is contested, although it has been shown that the majority of hypervirulent isolates possess copies (both chromosomally and on plasmids) of both the *rmpA* and *rmpA2* genes (positive regulators of capsule production). The presence of both these genes is uncommon in capsular serotypes not associated with hypervirulent isolates (46).

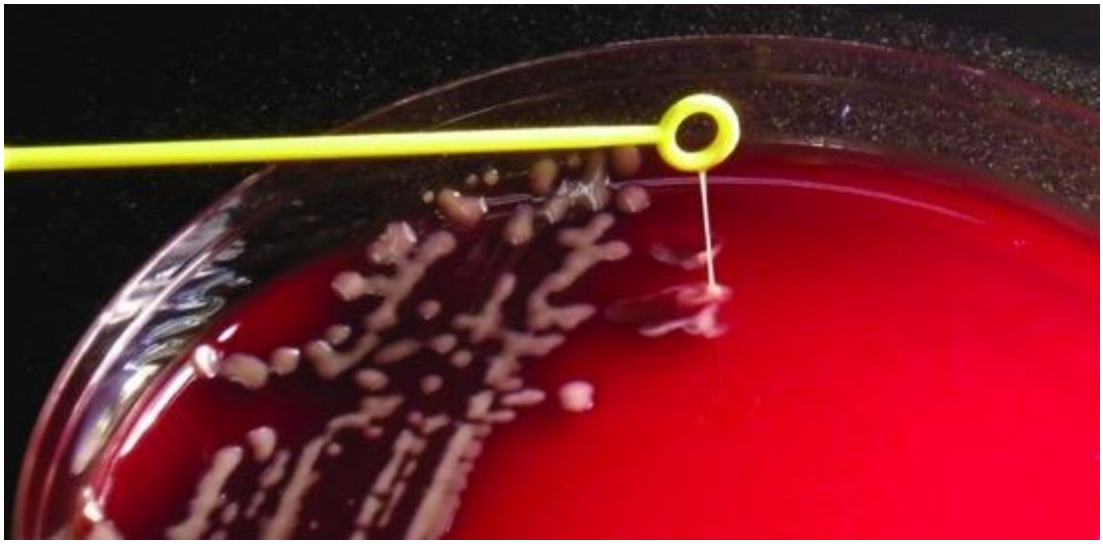


Figure 6 Positive "string test" on a hypervirulent strain of *K. pneumoniae*
String test is deemed positive if the dipping of a loop into a culture can produce a viscous string of more than 5 mm (44).

Hypervirulent *K. pneumoniae* isolates, in general, are more susceptible to antibiotics than their "classical" counterparts, with speculation that their higher levels of capsule expression make them less able to acquire plasmids or that antibiotic resistance genes have been lost in these isolates when they become hypervirulent (47). However, studies from China have shown resistance to antibiotics is increasing over time, with carbapenems being the only antibiotic some hypervirulent isolates show sensitivity to (47). With this in mind, hypervirulent *K. pneumoniae* has the potential to become the next "super-bug".

3. Antibiotic resistance

Antibiotics have been in use for over 60 years and have long been considered the panacea for infectious disease and hence have been extensively misused in the treatment of both humans and in food-producing animals. In Alexander Fleming's Nobel Prize speech in 1945, awarded for the discovery of penicillin, he warned of the development of antimicrobial

resistance and already by the 1950s penicillin resistant *Staphylococcus aureus* was common place in hospitals (48). Since that time antibiotic resistance has increased to the extent that bacteria commonly causing infections in hospitals and the community show resistance of 50% or more to commonly used antibiotics across the globe (Table 4).

Name of bacterium/ resistance	Examples of typical diseases	No. of 194 MS providing national data	No. of WHO regions with national reports of 50 % resistance or more	Range of reported proportion of resistance
Escherichia coli	Urinary tract infections, blood stream infections			
-vs 3rd gen. cephalosporins		84	5/6	0-82
-vs fluoroquinolones		90	5/6	3-96
Klebsiella pneumoniae	Pneumonia, blood stream infections, urinary tract infections			
-vs 3rd gen. cephalosporins		85	6/6	2-82
-vs carbapenems		69	2/6	0-68
Staphylococcus aureus	Wound infections, blood stream infections			
-vs methicillin "MRSA"		83	5/6	0.3-90

Table 4 Bacteria commonly causing infections in hospitals and communities
From WHO Antimicrobial Resistance Global Report on Surveillance 2014

Along with increasing antibiotic resistance there has been a paucity of new antibiotics coming into use, with no new antibiotic classes discovered since the late 1980s (Figure 7). The development of new antimicrobials is high cost and antimicrobials do not constitute a good investment for pharmaceutical companies as the public health policy to limit the usage of antibiotics in order to preserve their efficacy runs in direct opposition of traditional sales-based models of cost recovery (49).

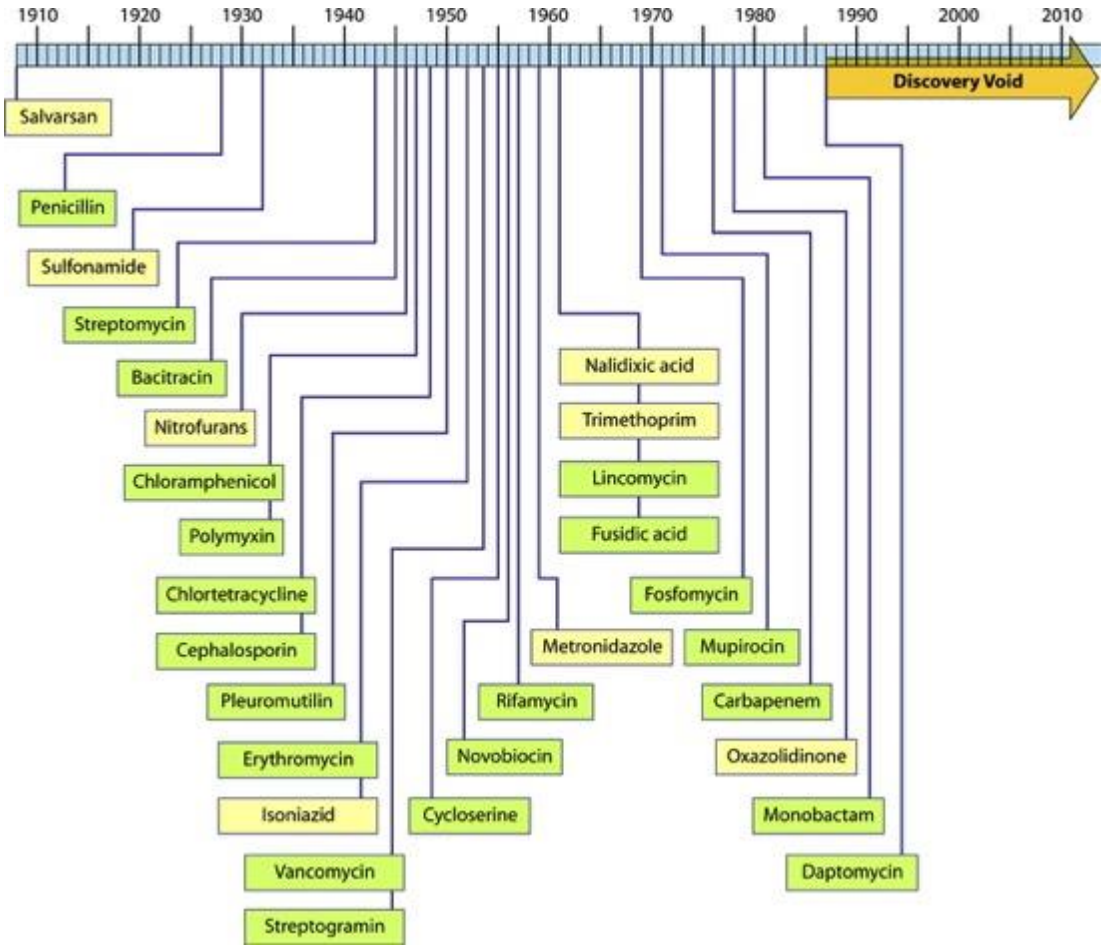


Figure 7 Dates of discovery of distinct classes of antibacterial drugs
From Silver et al. 2011 (50)

The evidence that antibiotic resistance exists in the environment and is hard wired into the microbial pangenome is increasing with studies from isolated environments such as the Lechugiilla caves in New Mexico showing 65% of Gram-negative isolates were already resistant to 3-4 commonly used antibiotics (51). With this in mind, development of resistance seems inevitable with the inappropriate use of antibiotics helping drive selection of genes mediating resistance.

Antibiotic resistance is becoming an increasing issue for both *E. coli* and *Klebsiella spp.*

4. Antibiotic failure

Inappropriate antibiotic treatment (i.e. where the pathogen shows no sensitivity *in vitro* to the prescribed agent) is a significant issue in the management of infection. Inappropriate antibiotic treatment is estimated to be between 15 % for community-acquired infections and reaching as high as 35 % in nosocomial infections admitted to ICU (52). When initial empirical antimicrobial therapy fails in patients in septic shock there is an associated reduction in survival of approximately five-fold (55% to 11%); this rise in mortality risk applies to both Gram-positive, Gram-negative and *Candida* species (53).

With the spread of antibiotic resistance treatment failure is likely to become a more frequent occurrence. Resistance to last line antibiotics such as colistin and tigecycline is emerging and infections for which there is no treatment option available are starting to occur, especially in *E. coli* and *Klebsiella* infections (54-56).

The possibility of a “post-antibiotic era” speculated about in the media over the last few years might soon become a reality if new treatment options are not developed.

C. Neutrophils and phagocytosis

Elie Metchnikoff, holder of the epithet “the father of natural immunity”, who pioneered research in phagocytosis, first coined the term phagocyte in 1883: a fusion of the Greek word *phago* “*eating*” and the New Latin *cyta* which takes its origin from the ancient greek kútos meaning “*vessel or jar*”. Metchnikoff’s description of the role of phagocytes still holds true to this day:

“The broad fact that the invasion of the organism by microbes most often induces, on the one hand, an inflammatory reaction with its associated emigration of leukocytes, and that, on the other hand, the phagocytes are capable of including and destroying the invaders, leads us to admit that the afflux of phagocytes to the invaded region and their bactericidal properties are mechanism which serve to ward off bacterial attack and to maintain the integrity of the organism” (57),.

Neutrophils, a subclass of granulocytes, are professional phagocytes and the most abundant of all the leukocytes constituting 40 – 75 % of the circulating population of leukocytes in humans. Neutrophils, like all myeloid cells, derive from the bone marrow where neutrophil production constitutes the primary function (by volume) of the bone marrow, with basal rate estimated at 5×10^{10}–10×10^{10} neutrophils/day. Stages of maturation of neutrophils in bone marrow is summarised in Figure 8. Estimates of the half-life of neutrophils in the circulation vary significantly and can also be effected by disease state; estimates range from a few hours to over five days, although the study reporting a value of five days has received some criticism for its

techniques used, and a more conservative estimate sits at around six to eight hours (58, 59).

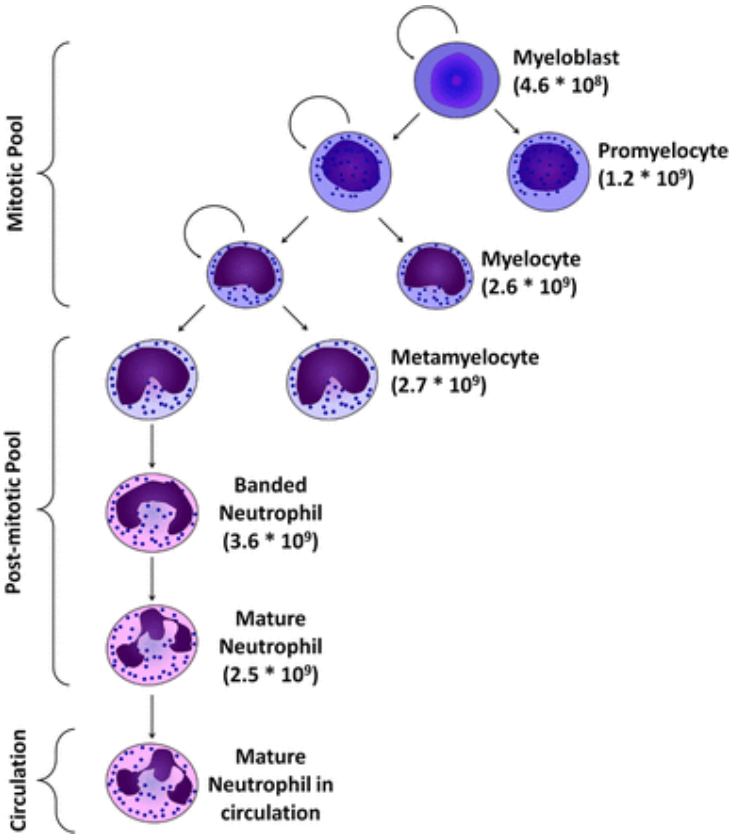


Figure 8 Production of the neutrophil lineage in the bone marrow.
Numbers in brackets represent total amount of each cell type per kg bodyweight. From Tak 2013 (58).

1. Neutrophils during infection

Neutrophils are a crucial component of the host immune defence; patients with a neutrophil count of less than 0.5 x 10^9 /L or defects in phagocytosis have an increased risk of recurrent and severe bacterial or fungal infections (often from uncommon pathogens) as well as poor wound healing, skin and deep visceral abscesses (60, 61).

a) Neutrophil recruitment

The recruitment of neutrophils to the site of infection involves a cascade of events initiated by interactions between bacteria and host cells (62). The receptors that facilitate neutrophil infiltration are tissue specific; Figure 9 details the process in the lower respiratory tract and how the actions of the neutrophil can be a double-edged sword, facilitating clearance of infection but also causing tissue damage to the host (62-64).

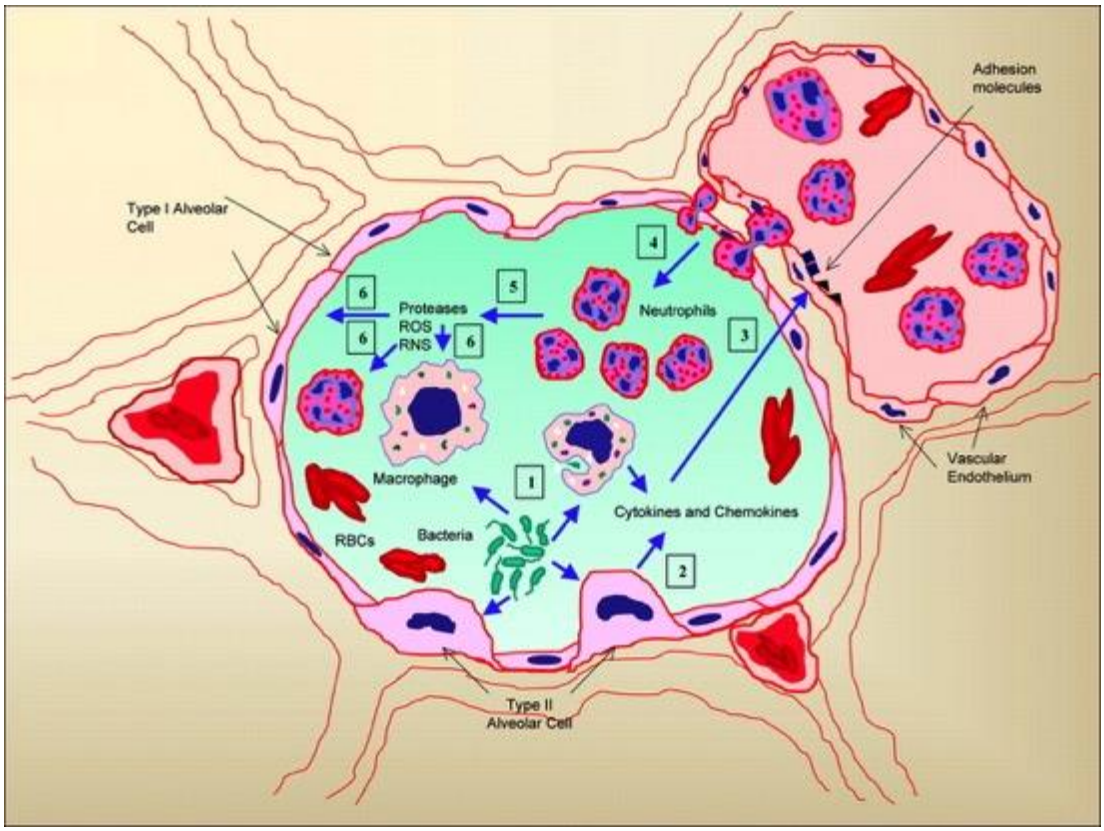


Figure 9 Schematic describing the cascade of events by which bacteria induce neutrophil infiltration and tissue damage in the lung
Bacteria first interact with epithelial cells and macrophages within the respiratory airway (1), this induces release of cytokines and neutrophil chemoattractants (2). Adhesion molecules on capillary endothelial cells are upregulated (3) and allow the transmigration of neutrophils into the alveolar spaces, following the chemotactic gradient (4). Neutrophils produce ROS and RNS (5), this can lead to necrotic cell death (6) and lung injury. Taken from *Craig et al*. 2009

A myriad of chemoattractants are capable of recruiting neutrophils to the site of infect, a list of these is detailed in Table 5. With such an array of chemoattractants showing the capacity to recruit neutrophils one may expect a degree of redundancy yet studies in inflammatory diseases have shown a temporal and spatial pattern of expression and an ability for neutrophils to prioritise chemotactic signals for efficient tissue homing; for example C5a, C3a and formylated peptides (which are found closer to the foci) are prioritised over IL-8 which is found more distally from sites of infection (65, 66).

Chemokines				
Systematic	Human	Murine	Human Receptor	Murine Receptor
CXCL1	GROα	KC	CXCR2	CXCR2
CXCL2	GROβ	MIP-2	CXCR2	CXCR2
CXCL3	GROγ	n/a	CXCR2	n/a
CXCL5	ENA-78	LIX	CXCR2	CXCR2
CXCL6	GCP-2	n/a	CXCR1/CXCR2	n/a
CXCL7	NAP-2	NAP-2	CXCR1/CXCR2	CXCR2
CXCL8	IL-8	n/a	CXCR1/CXCR2	CXCR2
CCL3	MIP-1α	MIP-1α	n/a	CCR1
CCL5	RANTES	RANTES	n/a	CCR1
CCL6	(MPIF-1)	C10	n/a	CCR1
CCL7	MCP-3	MARC	n/a	CCR1
CCL9	(HCC-2)	MIP-1γ	n/a	CCR1
CXCL12	SDF-1α	SDF-1α	CXCR4	CXCR4
Peptides/Cytokines				
			Receptor	
C5a			C5aR	
C3a			C3aR	
Formylated peptides (e.g. fMLF)			FPR1	
Pro-Gly-Pro (PGP)			CXCR2	
LL37			FPR2	
MIF			CXCR2	
Eicosanoids				
			Receptor	
Leukotriene B4 (LTB4)			BLT1	
Platelet activating factor (PAF)			PAFR	

Table 5 Major human and murine neutrophil-active chemoattractants and their receptors expressed on neutrophils. Adapted from Sadik 2011 (65)

Chemoattractants, along with microbial products, chemokines and inflammatory cytokines prime neutrophils, increasing phagocytosis efficiency

and ROS production in order to respond to infectious insults effectively (67). Priming of neutrophils increases expression of CD11b, CD35 FcγRs and CD66b, all thought to be released from intracellular stores via the exocytosis of secretory vesicles, without the need for *de novo* synthesis, allowing for rapid (10-15 min) responses to infection (68-72). Inflammatory cytokines also augment respiratory burst by phosphorylating NADPH oxidase components and deployment of flavocytochrome b_{558} from granules to plasma and phagosomal membranes (73-78). There is evidence from inflammatory conditions that a truly primed neutrophil also requires the expression of CD54 dectin-2 and IL-1β promoter activation, which necessitates transcription of proteins and therefore is less rapid and takes several hours (67).

b) Neutrophil – Bacterial killing

Neutrophils utilise three methods for bacterial killing: phagocytosis, degranulation and the release of neutrophil extracellular traps (NETs).

(1) Degranulation

Degranulation involves the release of granules containing proteins capable of killing microbes and digesting tissue: these are azurophilic (primary) granules, which contain myeloperoxidase (MPO), specific (secondary) granules, which contain lactoferrin, and gelatinase and (tertiary) granules, which contain matrix metalloproteinase 9. The production of granules marks the transition from myeloblast to promyelocyte with different granules appearing throughout development; specific granules occurring during the myelocyte and metamyelocyte stages are followed by the appearance of gelatinase granules in band cells (79). Degranulation can be triggered by

stimuli such as exposure to microbial products, binding of immune complexes, exposure to TNF-α and is regulated by interactions with cellular adhesins (80-82).

(2) Neutrophil extracellular traps

Neutrophil extracellular traps (NETs) are extracellular strands of decondensed DNA in complex with histones and granule proteins released by neutrophils. Extracellular trap formation has also been shown in mast cells, eosinophils, basophils, fibrocytes, macrophages and monocytes (83-88). This release of DNA, histones and granule proteins is effective in immobilising and killing some bacteria although it has been shown to be detrimental in other infections, for example the *Pseudomonas aeruginosa* exotoxin pyocyanin induces NETosis, which has been shown to play a role in the chronic inflammatory condition found in the cystic fibrosis lung (89) (90). NET formation has also been shown to have some protective effects during experimental systemic viral infections (91); whether NETosis is beneficial or not to the host depends on the anatomical site and infecting pathogen (90). Initially NETosis was considered to be a pathway of neutrophil cellular death, distinct from apoptosis and necrosis however a study in *Staphylococcus aureus* infection has now shown that NETosis can occur whilst the neutrophil maintains viability (92). NETosis has also been shown to be regulated by the size of the pathogen encountered; small pathogens such as bacteria induce phagocytosis whereas larger pathogens, which neutrophils would not be able to phagocytose, such as fungal hyphae or bacterial aggregates induce NETosis (93). Induction of either the phagocytosis or the NETosis pathway results in the inhibition of the other pathway (93). Excessive NETosis can

have a harmful effect during infection; the levels of cell free DNA (a surrogate marker for NET formation) correlates with sepsis severity and organ dysfunction as they contribute to tissue damage and histones can disrupt the function of some anticoagulants, which could effect the efficacy of prophylactic anticoagulants (94) (95).

(3) Phagocytosis

The first step in phagocytosis is recognition of the target to be phagocytosed, phagocytes possess an array of receptors for this purpose listed in *Table 6*. Pattern-recognition receptors can bind directly to bacterial and fungal proteins and sugars whereas the opsonic receptors recognise an opsonin, which is in turn bound to the bacterial or fungal surface (96).

Table 6 Human phagocytic receptors Adapted from Freeman et al 2014 (96)

Opsonic receptors	Pattern-recognition receptors
FcγRI (CD64)	Dectin-1 (CLEC7A)‡
FcγRIIA (CD32a)	CD14
FcγRIIC (CD32c)	Mannose receptor
FcγRIIIA (CD16a)	BAI1
FcαRI (CD89)	CD36
FcεRI	MARCO
CR1	Scavenger receptor A (CD204)
CRIg	
CR3 (αMb2, CD11b/CD18, Mac-1)	
CR4 (αXb2, CD11c/CD18, gp150/95)	
α5b1 (VLA-5)	

Opsonic and pattern-recognition receptors work in concert to facilitate phagocytosis. For successful phagocytosis remodelling of the cytoskeleton is required in order to engulf the particle in question; this is influenced by signalling from cell surface receptors where a signalling threshold must be met, otherwise stalling of phagocytosis can occur. This threshold has been

linked to the density of FcγR signalling in the early stages of phagocytic cup formation and 3′ phosphoinositide concentrations in later stages but is also influenced by properties of the target such as shape and rigidity (97-99).

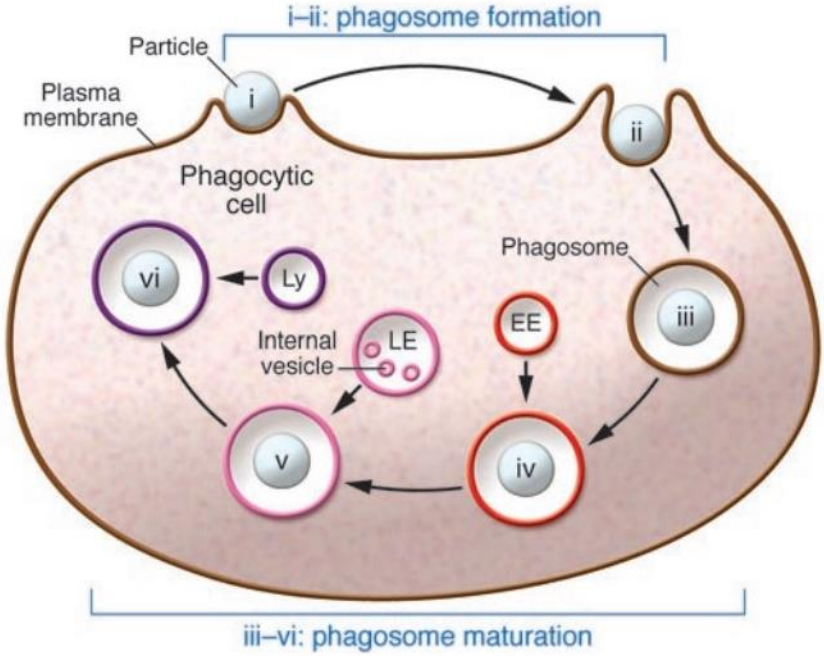


Figure 10 Phagosome formation and maturation
i. Particle engagement, ii. Phagocytic cup formation and iii. Nascent phagosome
After ligation of FcγRs the first detectable signalling event occurs: rapid phosphorylation of tyrosine residues within an immunoreceptor tyrosine-based activation motif (ITAM) domain, these are located either in the cytoplasmic tail of the receptor (CD32A) or in the associated homodimeric g-subunit (other FcγR) (100). This phosphorylation is mediated by Src- family tyrosine kinases (101). FcγR phosphorylation also promotes clustering of FcγR, which improves the efficiency of phagocytosis (102). ITAM-domain tyrosine residues form docking sites for Src homology 2 (SH2) domain-containing proteins. Syk is of particular importance to ITAM-dependent phagocytosis (101). This leads to activation of Rho GTP-binding proteins. FcγR-dependent engulfment requires Cdc42 and Rac2 (Rho-family proteins), whereas CR3 requires RhoA (without the requirement of tyrosine phosphorylation), both require RhoG (103). Arp2/3 is recruited and binds directly with WASP (Wiskott-Aldrich Syndrome Protein) and Scar/WAVE (Suppressor of cAMP receptor/WASP family Verprolin-homologous) proteins which act as nucleation points for actin (104) In addition to actin recruitment the surface area of the phagocyte increases during phagosome formation through the recruitment of recycling endosomes, late endosomes, endoplasmic reticulum and secretory vesicles to the site of phagosome cup (104). The actin forms pseudopod extensions, which engulf the particle, followed by the shedding of cytoskeleton proteins leaving the phagosome free in the cytosol (105).
iv. Early phagosome (2-10 min after sealing), v. late phagosome (10–30 min after sealing, vi. phagolysosome (>30 min after sealing).
Adapted from *Steinberg et al.* 2008 (106)

After sealing of the phagosome there is sequential fusion of early (EE), late (LE) endosomes and lysosomes (LY) with each step marked by the recruitment of specific molecules and a decline in pH:

Organelle	**Markers**
early endosome; early phagosome	EEA-1, Rab5, PI(3)P, syntaxin-13, transferrin receptor, VAMP3 pH 6.1
Late endosome; late phagosome	Rab7, Rab9, mannose-6-phosphate receptor, syntaxin-7, LAMPs, LBPA pH 5.5 –pH 6.0
Lysosome; phagolysosome	LAMPs, mature cathepsin D; fluid-phase markers chased for ≥2 hr pH 4.5 – pH 5.5
Adapted from *Scott et al*. 2003 (107)	

This process results in phagoslysosome with a very low pH, hydrolytic enzymes for particle digestion, defensins and other bactericidal peptides, and the ability to generate toxic oxidative compounds (108).

(a) Fc gamma receptors (FcγRs)

FcγRs, so named, as these are the receptors that bind to the Fc portion of IgG, are key to opsonophagocytosis. FcγRs form the bridge between the innate and adaptive immune response, utilising IgG produced via an adaptive immune response to react to an infectious insult. In humans there are four FcγR types that participate in phagocytosis: FcγRI (CD64), FcγRIIA (CD32a), FcγRIIC (CD32c), FcγRIIIA (CD16a) (109). FcγRIIIB (CD16b) is another FcγR that does not directly contribute to opsonophagocytosis but is constitutively expressed by neutrophils and has a role in cell activation, Ca^{2+} release and cross-linking of this receptor has been shown to induce NETosis (110, 111). In mice, only FcγRI (CD64), FcγRIII (CD16) and FcγRIV have been identified; receptor affinities for IgG are show in Figure 11(109).

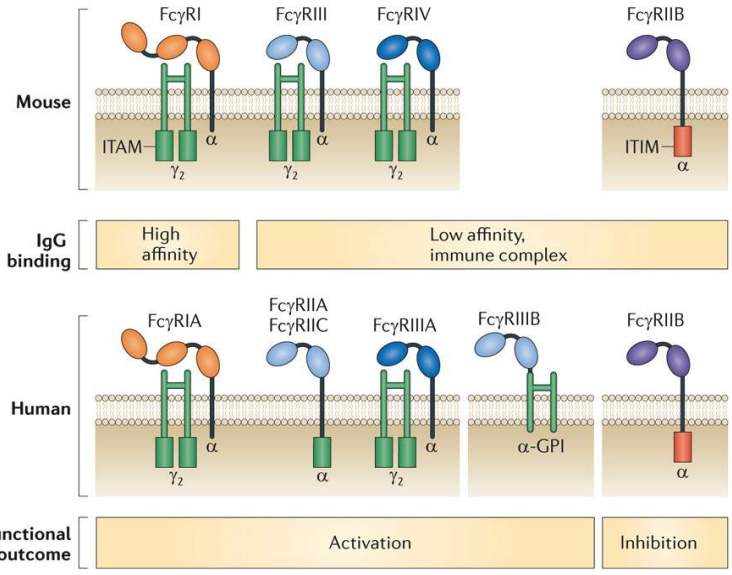


Figure 11 Comparison of human and murine FcγRs
FcγRs found on both humans and mice cells, their IgG binding affinity and whether binding of immune complexes results in activation or inhibition of cell function. Adapted from Schwab et al. 2013 (112)

In addition to the receptors mentioned above, FcγRIIB (CD32b), an inhibitory receptor, belongs to this class. CD32b is not thought to be expressed on

human neutrophils with its expression limited to B cells; CD32b's role being the negative regulation of antibody production (109). In mice, however, CD32b is expressed on neutrophils (109).

(b) Antibodies

Antibodies are a major component of humoral immunity and are one of the most abundant protein components of the blood, making up about 20% of the total protein in plasma by weight. Patients with primary antibody deficiencies are more susceptible to infection and are often treated with immunoglobulin replacement therapy with products such as intravenous immunoglobulin (IVIG) (113)

In humans antibodies exist as 5 classes: IgA, IgD, IgE, IgG and IgM. IgG is important in opsonophagocytosis as it can interact with FcγRs (as well as glycan binding receptors DC-SIGN and CD23) and is capable of inducing antibody-dependent cellular phagocytosis as well as complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity (96). The structure of IgG can be split into two parts: the Fc region, which binds to FcγRs and the Fab region, which is responsible for recognising and binding antigen.

(c) Complement and complement receptors

Complement can act as an opsonin or can lyse pathogens directly via the membrane attack complex. Complement can become active and interact with pathogen surfaces in a number of ways: the classical, alternative or lectin pathway, each is briefly detailed in Figure 12.

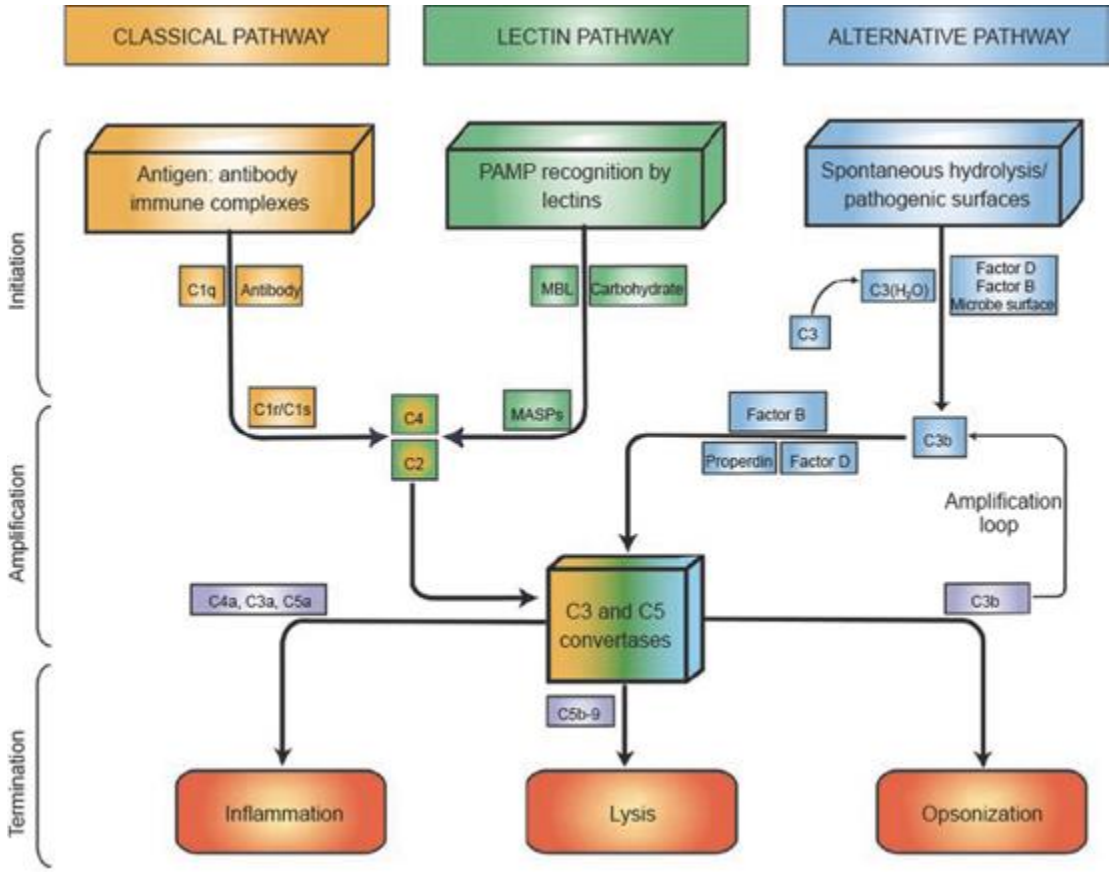


Figure 12 Complement system pathways
The classical pathway activates with binding of C1q to antibody:antigen immune complex; the lectin pathway activates when mannose-binding lectin (MBL) binds to conserved microbial carbohydrate motifs resulting in the activation of MBL associated serine proteases – both of these pathways lead to in the cleavage of C4 and C2 to form C3 and C5 convertases. Alternative pathway activation results from the spontaneous hydrolysis of C3 forming the alternative pathway C3 convertase. C3 and C5 convertases generate the key effectors of the complement system: opsonins (C3b), anaphylatoxins (C3a, C4a and C5a) and the membrane attack complex. Taken from Dunkelberger et al 2009 (114)

C3b binds to complement receptors CR1, CR3 (CD11b/CD18, integrin aMb2) and CR4 (CD11c/CD18, integrin aXb2) and the recently discovered CRIg (found primarily on Kupffer cells) (114). As well as participating in phagocytosis, the complement and Fcγ receptors can modulate the expression of one another, for example: activation of C5aR (CD88) leads to an increase in the expression of CD16, which is protective in *Pseudomonas aeruginosa* infection; CD88 knockout mice showing a similar phenotype

during *P. aeruginosa* infection (increased susceptibility) to CD16 knockout mice (115).

2. Immune dysfunction

Immune dysfunction is a defining feature of sepsis and plays a major role in the pathophysiology of the disease. Historically, sepsis has been thought of as a condition with two distinct phases: the initial hyper-inflammatory phase followed by a hypo-inflammatory/immunosuppressive stage. Recent studies now suggest that both occur simultaneously with the net initial effect being hyper-inflammatory (116). There are however competing views as to which aspect causes death in patients.

Figure 13 details two theories regarding immune responses in sepsis; theory 1 describes a model in which early deaths are caused by excessive inflammation whereas late deaths are a result of a failure to clear initial infection or the acquisition of secondary infection due to persistent immunosuppression (117). Theory 2 describes a model in which protracted inflammation driven by the innate immune system leads to organ dysfunction and that patients who die, although suffering impaired adaptive immunity, die due to the longer and more severe organ injury resulting from persistent innate immune driven inflammation (118). Data from post-mortems on patients who died from sepsis give greater support to theory 1; patients showed decreased production of pro- and anti-inflammatory cytokines, up regulation of inhibitory receptors (including PD1), increases in regulatory T cell and myeloid-derived suppressor cell populations, and down regulation of CD28 and HLA-DR-mediated activation pathways (119). It has also been

shown that the rate of positive blood cultures increase in late stages of sepsis along with an increase in the number of common opportunistic pathogens detected (120).

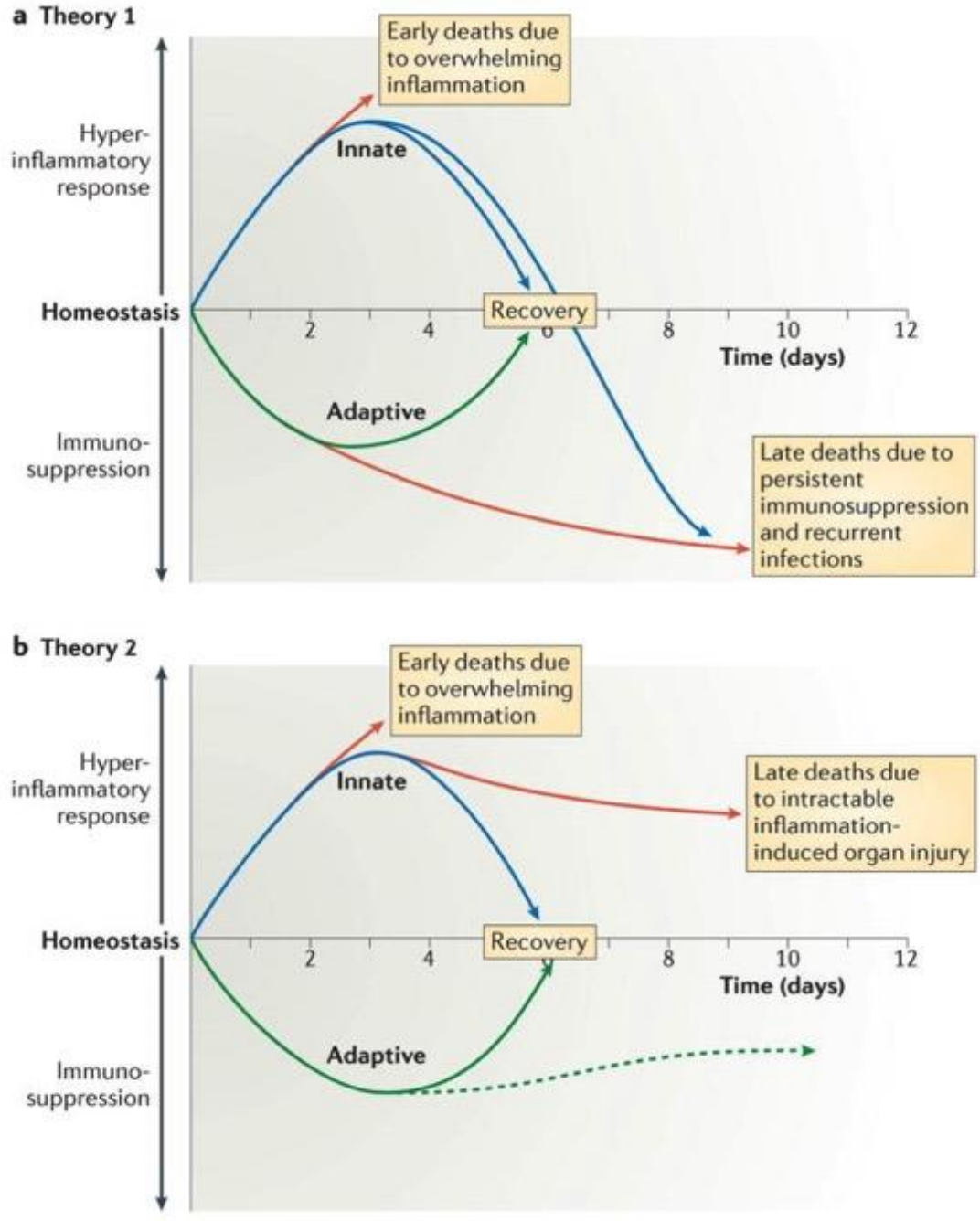


Figure 13 Competing theories of host immunity in sepsis
From *Hotchkiss et al*. 2013 (116)

Further evidence to support theory 1 includes the results of surgical sepsis patient post-mortems, which showed that 80% of patients had evidence of an infectious focus still present at death (121).

The two-year mortality of patients who survive an initial bout of severe sepsis is 1.5 fold higher than other hospitalised patients with mortality varying between studies from mortality of 44.9 % to as high as 67 % (122). This increase in mortality in survivors is attributed to long-term (nine months – five years) sepsis-induced impairment of immune responses: exhibiting both a low-level inflammatory status and stunted cytokine production in response to inflammatory stimuli, leaving patients more susceptible to subsequent infections (123).

a) Neutrophil dysfunction

Neutrophil dysfunction is an important factor in the pathophysiology of sepsis. Many new treatments for sepsis have been aimed at modifying neutrophil responses during sepsis. Severe infection leads to release of immature weakly active neutrophils from the bone marrow with decreased phagocytosis, decreased chemotactic capability, decreased oxidative burst and an increased production of IL-10 (124). Chemotaxis of neutrophils in sepsis is impaired to the degree that it has been proposed as possible means of evaluating sepsis severity and in major burns (>20% body surface area, full thickness burns), where sepsis diagnosis is complicated by excessive inflammation, changes in neutrophil migration patterns can predict sepsis up to two days before sepsis diagnosis is confirmed (125, 126). Some of this neutrophil dysfunction has been shown to be mediated by C5a.

Neutrophil activation by C5a can result in the blocking of activation of other neutrophils by triggering the release of serine proteases; these then cleave CD88 from surrounding neutrophils (127). This is thought to prevent excessive neutrophil activation in the context of a small, localised infection, but has been implicated in neutrophil dysfunction in both sepsis and the cystic fibrosis lung (127). As well triggering the cleavage of CD88, C5a binding also inhibits the activity of RhoA and hence polymerization of actin and phagocytosis (128, 129). CD88 expression is being explored as a possible marker of infection severity in sepsis as reduced expression (due receptor cleavage or possibly internalisation) correlates with increased infection severity and poorer outcomes in patients (130). This reduction in CD88 expression was coupled with a reduction in IL-8 production by neutrophils in sepsis patients when stimulated with C5a (130). Anti-C5a therapies have been trialled in animals with successfully showing protective effects in experimental sepsis but none have yet been developed as far as human trials (131-133).

Due to the capability of neutrophils to produce ROS and NETs they also pose a risk to the host; NETosis as been associated with organ dysfunction and hence therapies to induce IL-10-mediated inhibition of neutrophil infiltration into tissue (95, 134)

3. Sepsis Treatment

Patients with sepsis and other severe infections present a significant challenge to treatment, often presenting with a diverse range of associated co-morbidities and identifying the etiologic agent is often unachievable (135).

Over recent years steps have been taken to improve the outcome in severe CAP (outlined in the Surviving Sepsis Campaign), yet mortality remains high (136). New treatment strategies are therefore urgently needed to complement the existing options currently available and limited to antibiotics, organ support and source control (137).

a) Antimicrobials

"Frapper fort et frapper vite" Paul Ehrlich, 1913

(Hit hard and fast)

Since 2002 when the Surviving Sepsis Bundles (Table 7) were introduced there has been a movement towards early goal-directed therapy (138). Now these bundles have been in place for a number of years it has been shown that of the measures introduced only rapid administration of antibiotics has had an effect on increasing survival above that of normal care, whilst the efficacy of the other measures is controversial (138). Each hour of delay in administration of antibiotics is associated with a 7% increase in mortality (139). These improvements of course depend on the antibiotic being administered being effective against the pathogen being treated, with antibiotic resistance increasing failure of antibiotics is possibly going to become more common.

Table 7 Sepsis bundles.

Resuscitation bundle (to be achieved within 6 hours from severe sepsis/septic shock diagnosis)	
1 - Measure blood lactate	
2 - Blood cultures	*At least 2 sets of blood cultures before administration of antibiotics*
3 - Antibiotics	*Broad-spectrum antibiotics within 3 hours of admission to the emergency department or within 1 hour of admission to other hospital units*
4 - SvO_2	*Measure and achieve central venous oxygen saturation >70%*
5 - Fluid resuscitation	*If hypotension and/or blood lactate >4 mmol/L*, *1 L crystalloids (or 0.5 L of colloid equivalent) in 30 minutes*
6 - Central Venous Pressure	*If hypotension despite fluid resuscitation and/or blood lactate >4 mmol/L*, *achieve CVP >8 mmHg*
7 - Vasopressors	*If hypotension not responding to fluid resuscitation*, *maintain a mean arterial pressure >65 mmHg*
Management bundle (to be achieved within 24 hours from severe sepsis diagnosis)	
1 - Lung protective ventilation	*Maintain inspiratory plateau pressures <30 cmH_2O for mechanically ventilated patients; avoid a tidal volume >6 mL/kg for patients with acute respiratory distress syndrome*
2 - Steroids	*Administer low-dose steroids for septic shock in accordance with a standardized hospital policy*
3 - Drotrecogin alfa (activated)	*In accordance with a standardized hospital policy* *(drug withdrawn from market* (140))
4 - Glucose control	*> 4 mmol/L but <8.3 mmol/L*

Adapted from Damiani *et al* 2015 (141)

b) Immune modulating treatments

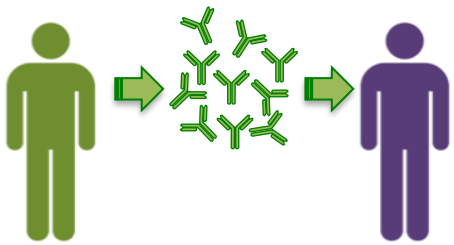
Attention is now shifting towards the use of immunomodulatory drugs as adjunctive therapies in infectious disease (142). Treatments trialled so far in both CAP and sepsis have primarily targeted the hyper-inflammatory phase: however statins, steroids and activated protein C have all failed to show significant clinical benefit (140, 143, 144). Following on from these failures more interest has developed in targeting immunosuppression during infection: recombinant GM-CSF, IL-7 and anti-programmed cell death 1 receptor (PD-1) antibodies are all currently undergoing trials (142). The results of phase II clinical trials in Germany on the use of GM-CSF in sepsis were reported in 2009 and showed patients treated with GM-CSF had reduced time of mechanical ventilation and shorter hospital/ICU stays (145). Phase III trials in France are currently recruiting for a study investigating the effects of GM-CSF on rates of infection in ICUs (146). IL-7 is currently in phase II clinical trials in the US investigating the ability of IL-7 to restore lymphocyte counts in sepsis patients (147). Anti-PD-1 antibodies are still in the pre-clinical stages of development but *in vitro* studies have shown that blockade of the receptor decreases apoptosis and improves immune cell function in septic patients (148). The anti-PD-1 antibody pembrolizumab, produced by Merck already has FDA approval for treatment of melanoma so could quickly progress to human trials (149).

New treatment strategies are desperately needed to complement the existing options available that are currently limited to antibiotics, organ support and source control (137).

D. P4 therapy

One possible new immunomodulatory treatment is P4 therapy; a combination passive immunotherapy along with the immunoactivating peptide P4.

1. Passive immunotherapy



Passive immunotherapy – the transfer of functional antibodies from an immunised host to a susceptible host

a) History

The transfer of antibodies as a treatment of infection has been used for over a century; early preparations used for passive immunotherapy were relatively crude, utilising serum from immunised animals and were associated with a high incidence of side effects, known as “serum sickness”, in patients (up to 50%) ranging from relatively mild symptoms such as itching and rashes to more serious symptoms such as hypotension and shock (150, 151). Despite the severe side effects observed, by the 1930s serum therapy had become the standard therapy for pneumococcal pneumonia, although this treatment required early administration, having little effect if administered after 4 to 5 days after the onset of symptoms. Passive immunotherapy, along with potentially severe side effects during the treatment of pneumococcal disease, was also hindered by serotype specificity - a mixture of serum, from the immunisation of animals with different serotypes, was required in order to

ensure efficacy in the absence of a specific diagnosis with regards to pneumococcal serotype, which more often than not was unknown at the time of treatment (152). Passive immunotherapy was also an expensive therapy to produce, as it required not only the animals used to produce the immune

serum but also required *in vivo* testing for efficacy and potency of each batch to determine treatment doses (152). Fleming's discovery of penicillin in 1928 and subsequent mass production of the drug pioneered by researchers lead by Howard Florey in the 1940s resulted in a decline in serum therapy in favour of penicillin with its broader specificity, more favourable side effect profile and cheaper production (152).

	Antibody Therapy		
	Immune Serum	**Human mAb**	**Chemotherapy**
Specificity	Narrow	Narrow	Broad
Source	Animals Humans	Tissue culture	Fermentation Chemical synthesis
Toxicity	High	Low	Low
Cost	High	High	Low
Administration	Difficult	Easy	Easy
Pharmacokinetics	Variable	Consistent	Consistent
Mechanism of action	Antimicrobial Immune enhancement Toxin neutralisation	Antimicrobial Immune enhancement Toxin neutralisation	Antimicrobial

Table 8. Comparison of immune serum, human monoclonal antibodies and chemotherapy for treatment of infection

Antibody based therapies have developed over the years as production techniques have improved and there are now an immense number of monoclonal antibody (MAb) therapies being utilised in other fields such as oncology and inflammatory disorders, yet usage in bacterial infection is limited. Table 8 compares immune serum, human MAb and chemotherapy (such as antibiotics) in the treatment of infection.

b) Intravenous immunoglobulin in passive immunotherapy

Intravenous immunoglobulin (IVIG) is already used in the treatment of several autoimmune and inflammatory diseases but has potential for use in infectious disease (153). Utilising IVIG for the treatment of severe bacterial infections has the potential to bridge the gap between human mAb and conventional chemotherapy by offering a treatment with broad specificity, low toxicity, easy administration, consistent pharmacokinetics and diverse antimicrobial actions. Unfortunately the use of IVIG in clinical trials in the treatment of sepsis thus far have show conflicting results, with outcomes varying depending on the dose and the IVIG preparation used in each study (154, 155). As well as inconclusive results in clinical trials, IVIG is an expensive drug with a cost of £20,850 per quality adjusted life year, just above the £20,000 limit for treatments on the NHS (156). It is also not totally understood how IVIG acts during treatment for sepsis as there are numerous components of the immune response to infection whose actions can be modified by the administration of IVIG (detailed in Figure 14) (112).

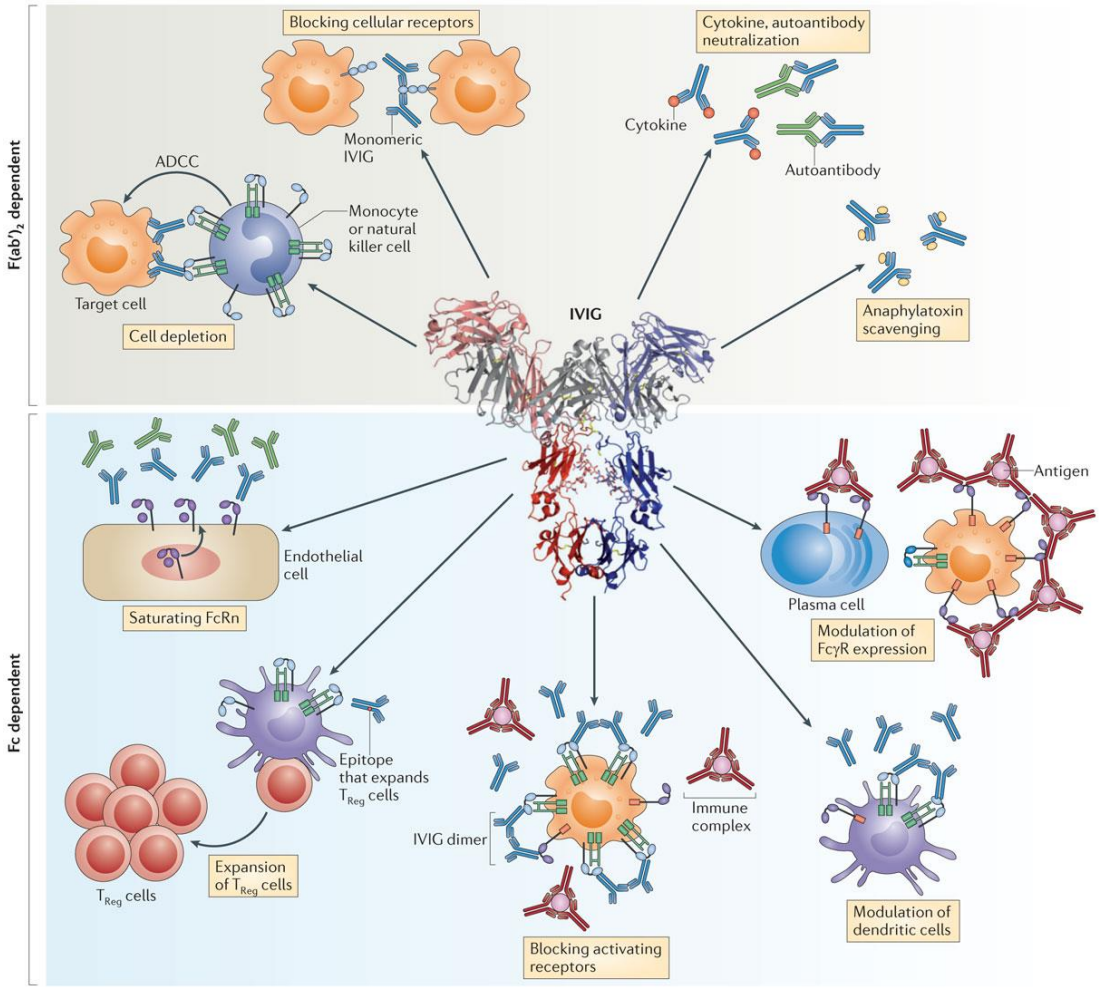


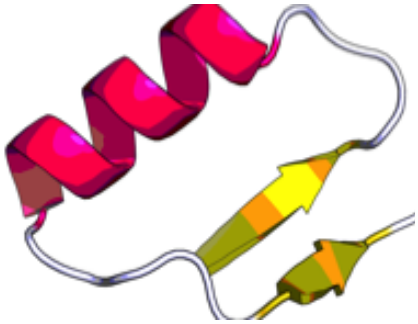
Figure 14 Fc and Fab mediated IVIG activity
The top panel shows the Fab mediated mechanisms of immunomodulation: antibody-dependent cytotoxicity, blockade of cell surface receptors, the neutralisation of cytokines and scavenging of anaphylatoxins. The bottom pane show Fc mediated mechanism of immunomodulations: the blocking/saturation of cell surface receptors (such as FcγR) , modulation of FcγR expression and expansion of T_{reg} cells. Taken from *Schwab et al*. 2013 (112)

Effectiveness of IVIG as an opsonin during passive immunotherapy is limited by the requirement for frequent large doses of antibody (at significant financial cost) and the capability of the host's cellular response to utilise the IgG for clearance of pathogens (157).

2. P4 peptide

The P4 is a 28 amino acid peptide derived from the lipoprotein pneumococcal surface adhesin A (PsaA), which is being investigated due to its immunomodulating effects.

PsaA is a highly conserved molecule, which is partially exposed on the pneumococcal surface, although the majority of the molecule is concealed by capsular polysaccharides. PsaA functions as a manganese transporter in the pneumococcus and is also a putative adhesin binding to E-cadherin (158).



- MW = 3254.8 Da
- Amino acid residues 251–278
- Amino acid sequence:

  L-F-V-E-S-S-V-K-R-R-P-M-K-T-V-S-Q-D-T-N-I-P-I-Y-A-Q-I-F

Figure 15. P4 peptide
Crystal structure, molecular weight and amino acid sequence of P4 peptide.

P4 peptide was developed when the Center of Disease Control and Protection were looking for the functional epitope within PsaA, which allowed binding of the pneumococcus to epithelial cells with the intention of then using that epitope to develop treatments targeted at blocking bacterial binding through PsaA. A series of peptides were produced based on the sequence of PsaA, those of interest were named P4, P6 and P7. P4 is homologous to the PsaA sequence with a conservative substitution of two amino acid: a pair of aspartic acid to a lysine and arginine (detailed in Figure

16) this substitution was outside of areas in the sequence thought to be of functional importance (159).

P6 and P7 are truncations of P4, with P6 being truncated at the N-terminus whilst P7 was truncated at the C-terminus (sequences shown in Figure 16). They found that fluospheres coated in both P4 and P7 had high binging to nasopharyngeal cells (Detroit 562) whereas P6 had low binding (159). They went on to treating nasopharyngeal cells with 20 µg/well of P4 peptide before adding 1 µm fluorescent polystyrene spheres (fluospheres) coated in rPsaA, which resulted in a 95.5% inhibition of the rPsaA fluospheres to nasopharyngeal cells (159).

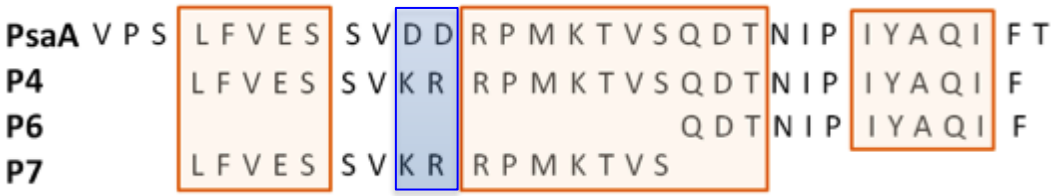


Figure 16 Comparison of P4, P6 and P7 sequence with PsaA sequence.
Amino acid sequences of PsaA, P4, P6 and P7 – orange boxes indicate areas thought to be functional domains of PsaA. Blue box indicates location of amino acid substitution.

When treatment with P4 was tried out on nasopharyngeal cells with live *S. pneumoniae*, rather than blocking binding, increased adhesion and invasion, electron micrographs of these cells showed that those treated with P4 had large vacuoles containing internalised material, which was thought to be suggestive of a strong cellular activation. Increases in adhesion/invasion were not serotype specific and were also observed with *Streptococcus pyogenes* and *Streptococcus mitis (160).*

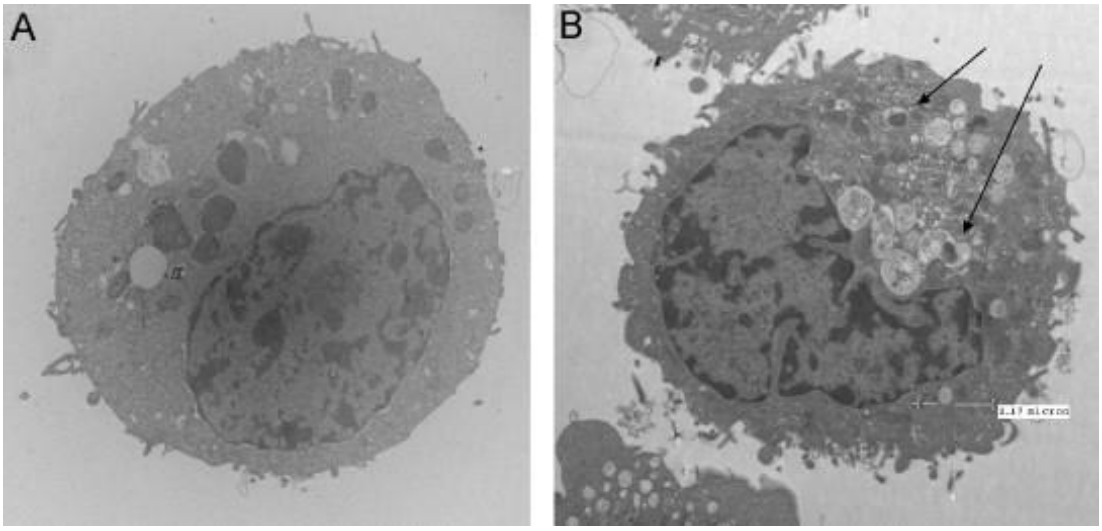


Figure 17 Transmission electron micrographs of P4-treated and non-treated Detroit 562 cells (1900×)
Panel A. Non-treated Detroit 562 cells. Panel B. Cells treated with P4. Arrows indicate large vacuoles containing internalised material (160).

This proposed cellular activation was also observed in undifferentiated human neutrophils (HL-60), mouse macrophages (RAW 261.4) and freshly isolated human blood neutrophils. In addition to cellular activation it was observed that P4 treatment led to an increase in basic fibroblast growth factor (FGF-β) and a reduction in IL-6, IL-8 and Vascular endothelial growth factor (V-EGF) secretion by nasopharyngeal cells. No differences were observed in the secreted levels of cytokines IL-1, IL-10, TNF-*α*, IFN-*γ*, and GM-CSF (160).

Following on from these studies P4 was used in opsonophagocytosis assays with HL-60, IVIG and complement to look at the effect of the peptide on phagocytosis of pneumococci. P4 significantly enhanced the phagocytosis of pneumococci by HL-60s in a dose dependent manner but its effect was dependent on the presence of antibody and complement as the response was mitigated by the removal of either two components (Figure 18) (161).

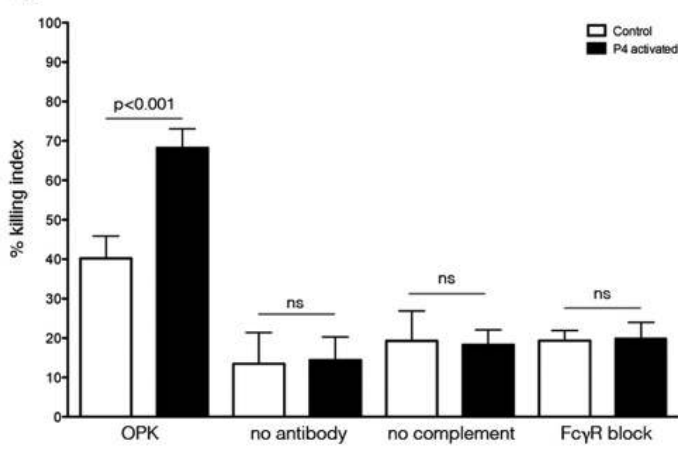


Figure 18 Effect of removing assay components from OPK on P4 enhancement of phagocytosis in human alveolar macrophages.
"OPK" = all components are included (macrophage, bacteria, antibody, complement), "no antibody" = without the presence of antibody, "no complement" = without the presence of complement, and "FcγR block" = where Fcγ receptors were occupied by IgG prior to the assay. Taken from (162)

Table 9. Published P4 in vivo research.

Year	Main findings
2008 (161)	P4 and antibody treatment via I.P. injection lead to 60% survival, I.V injection to 80% survival whilst untreated controls had only 10% survival
2009 (163)	Treatments with P4 and IVIG in combination with antibiotics reduced the dosage of antibiotic required and lead to increased survival when compared to antibiotics alone. Animals which survived pneumococcal pneumonia after treatment with P4 and IVIG were re-infected and treated a second time with P4 and IVIG – P4 still showed efficacy in repeat treatments.
2010 (164)	P4 treatment in two strains of aged mice was still effective against pneumococcal infection.
2011 (165)	Treatment with P4 and IVIG rescued mice from fatal *Staphylococcus aureus* infection with survival increased from 20% to 70%.
2011 (166)	Mice were challenged with influenza before infection with pneumococcus – P4 and IVIG treatment improved survival from 20% to 80%
2012 (167)	Mice infected with pneumococcus were treated with P4 and IVIG. Mice receiving an intranasal dose at an early time point (12 and 18 hrs) saw significant improvements in survival from 0% for controls to 100% for treated mice at 48 hours post infection. Mice that received a later dose intravenously (24 and 30 hours) saw significant improvements in survival from 0% to 60%.

Having shown *in vitro* efficacy at enhancing phagocytosis, studies moved on to *in vivo* models of infection. Animal studies so far have looked at treatment of *S. pneumoniae* (with and with out influenza) and *Staphylococcus aureus* with P4 peptide in combination with antibodies and have shown treatment lead to increased survival. These studies are summarized in Table 9. From the *in vivo* work it was established that treatment with P4 peptide led to an increase in the surface expression of CD32/16 (FcγRII/III) on both neutrophils and macrophages of mice both during infection in in naïve mice (167).

Human *ex vivo* studies showed that treatment of alveolar macrophages and peripheral blood neutrophils with P4 peptide in an OPK assay also resulted in increased phagocytosis of pneumococci in healthy volunteers from both the UK and Malawi (162). Although populations showed significant increases in phagocytosis, the baseline killing of macrophages from UK volunteers was higher and treatment led to greater improvements in phagocytosis than were seen in Malawian volunteers (162). As well as increased phagocytosis this study also showed increased intracellular oxidation; this increase in intracellular oxidation was only significantly increased during OPK assay however and not in cell treatment directly with P4 only (162). This study also showed no increases in inflammatory cytokines or markers of cellular activation on alveolar macrophages treated with P4 following an OPK assay (162). Based on these *in vitro* and *in vivo* studies a hypothesis for P4 mechanism of action was formed (represented in Figure 19); this hypothesis was that the enhancement in FcγR coupled with the administration of pathogen specific IgG (in the form of IVIG) leads to increased phagocytosis

of bacteria during infection and hence a decreased bacterial burden and improvements in survival. As of yet the mechanism by which P4 increases FcγR expression is unknown.

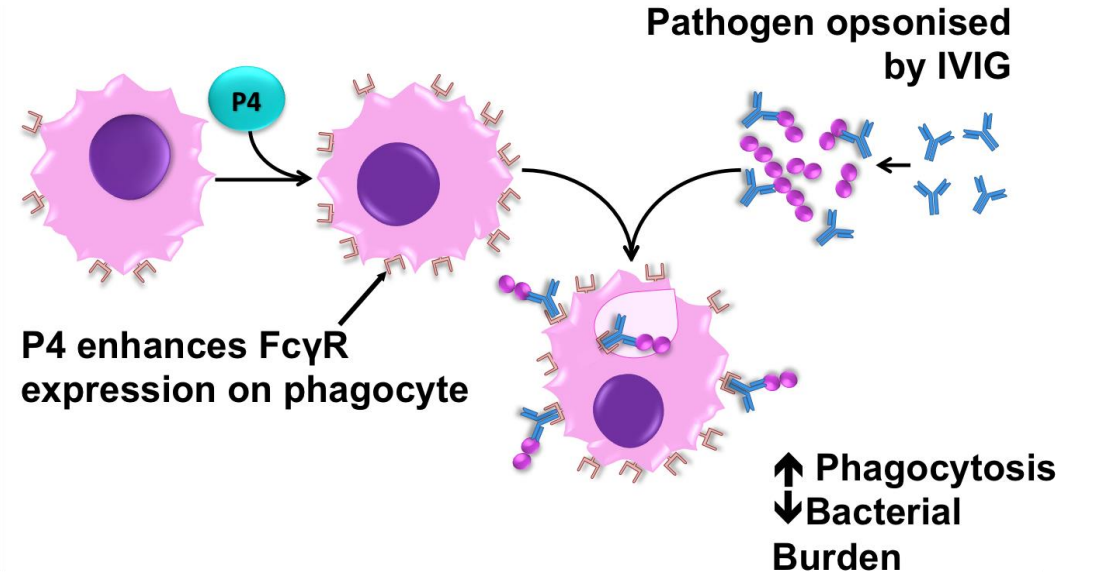


Figure 19 P4 enhances phagocytosis of IVIG opsonised bacteria through increased expression of FcγR

By using P4 with IVIG (P4-IVIG therapy) as the source of IgG, for opsonisation of pathogens, this treatment has the potential for use in numerous infections as IVIG contains antibodies specific to a wide range of common pathogens. This would be beneficial in the treatment of sepsis and other severe bacterial infections were the causative agent is often not known when treatment is initiated and patients are often suffering from depletion of immunoglobulins (168).

E. Aims and objectives

1. Gram negative infections

Having shown efficacy in the treatment of Gram positive infections (*S. pneumonia* and MRSA) with P4 peptide treatment, the first aim of this study was to explore the use of P4 in combination IVIG in the treatment of Gram negative infections, namely *E. coli* and *K. pneumoniae* most commonly associated with severe invasive infections such as sepsis. This will be achieved through the development and use of murine models of sepsis from which I will determine the effects of P4 treatment on bacterial load, neutrophil responses to infection, and overall inflammation and host survival patterns.

2. *Ex vivo* CAP

The second aim of this study was to assess *ex vivo*, the capacity of P4 peptide to enhance bacterial phagocytosis by peripheral blood neutrophils and alveolar macrophages in a cohort of patients admitted to ICU with community acquired pneumonia. As well as looking at phagocytic killing as a primary endpoint for the study I aimed to determine the effects of *ex vivo* P4 treatment on neutrophil cell surface receptor expression, inflammation and whether patient clinical measures would give an indication as to which patient groups would most likely benefit from P4 treatment.

3. Mechanism of P4 action

The third aim of this study was to investigate the effect of P4 on receptor expression on the neutrophils of naïve mice in an attempt to learn more about the mechanism of P4 function.

Chapter II. *In vivo* murine study of effects of P4 peptide during severe *E. coli* infection

A. Introduction

Previous studies have shown the *in vivo* efficacy of P4 peptide in the treatment of acute pneumonia and sepsis caused by the Gram-positive pathogens *Streptococcus pneumoniae* and *Staphylococcus aureus* (MRSA) (161, 164, 165, 167). This study goes on to look at the efficacy of P4 treatment in severe *Escherichia coli* infection.

1. Animal Model

For this study an animal model of *E. coli* peritoneal infection that rapidly progresses to sepsis was developed in order to assess treatment efficacy. This model was used for both survival and time pointed experiments. Models utilised also assessed the efficacy of P4 treatment as an adjunctive therapy in combination with antibiotic treatment. An intra-peritoneal infection model was chosen as this site is the third most common site of initial infection in sepsis, with treatment failure being associated with persistent or recurrent infection and high mortality.

2. Cell surface marker and cytokines

Tissue and blood from time pointed experiments were collected for flow cytometry analysis of cell surface markers and measurement of cytokines in order to monitor the effect of treatment on the immune response to infection.

The cytokines chosen for analysis were CXCL1, IL-10 and C5a. CXCL1 was chosen as it regulates the production of a number of cytokines, chemokines and adhesion molecules essential for neutrophil recruitment and activation as shown in Figure 20.

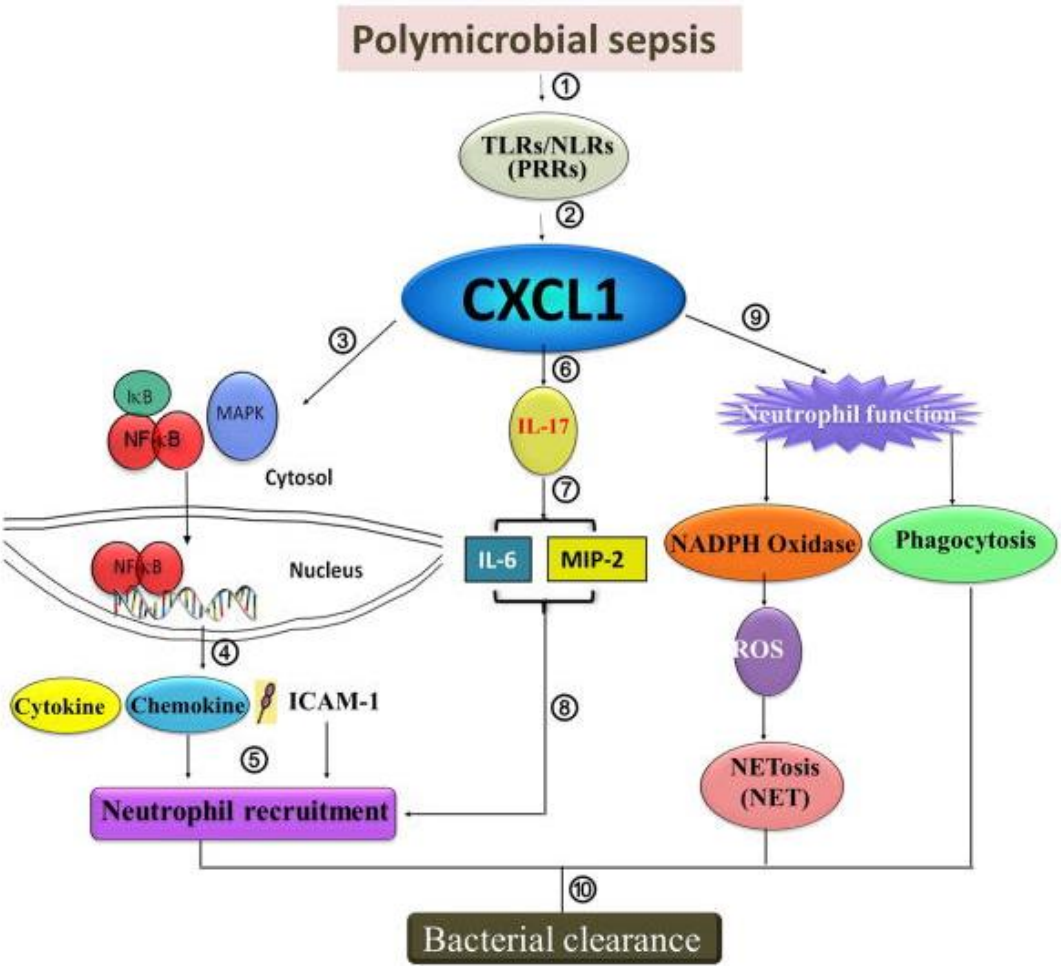


Figure 20 CXCL1 -mediated signaling cascades leading to bacterial clearance in the organs in response to polymicrobial sepsis
(1 and 2) CXCL1 production by hematopoietic and resident cells as a result of interaction with pattern recognition receptors. (3, 4 and 5) CXCL1 activates NF-κB, MAPK leading to upregulation of cell adhesion molecules (ICAM-1) and cytokines/chemokines, which results in neutrophil recruitment to the tissues from the bloodstream. (6, 7 and 8) CXCL1 regulates the production of IL-17 (IL-17A), resulting in the production of CXCL2/MIP-2 and IL-6 and hence neutrophil recruitment. (9) CXCL activates NADPH oxidase leading to the production of ROS, eventual NETosis and augmenting phagocytosis.(10) Enhanced neutrophil recruitment and activation leads to bacterial clearance. From Jin et al. 2014(169)

IL-10 was chosen as a cytokine of interest due the role it plays in immune modulation, both beneficial and detrimental effects have been reported for IL-10 in sepsis, on the one hand it can blunt the pro-inflammatory response,

reducing TNF and IFN-γ whilst on the other hand it has been shown to negatively effect splenocyte function (170-172). IL-10 has also been shown to play a major role in determining when animals enter a phase of irreversible shock and has shown to be protective in models of polymicrobial sepsis, with the timing of intervention being important and blocking of IL-10 being beneficial in the later stages of infection (173, 174).

C5a was measured due to the reported role in neutrophil dysfunction which was discussed in the introduction (Chapter I.C.2.a).

Cell surface markers investigated in this study included CD64, CD32/16, and CD88. CD64 and CD32/16; the FcγRs were measured as the proposed mechanism of P4 function is a modulation of FcγR expression. CD88 is the receptor for complement. CD88 is of interest because of the role of C5a-CD88 binding and its effects on neutrophil function as well as being a possible marker of infection severity.

B. Methods and Materials

1. Media Preparation

a) LB Agar

400 ml of distilled water was mixed with 8 g of Luria broth powder (Sigma, UK L3022) and 6 g of agar (Oxoid, UK LP0011) before autoclaving at 121°C for 15 minutes. Plates were poured once agar had cooled to 56°C. Plates were stored at 4°C until use.

b) LB Broth

400 ml of distilled water was mixed with 8 g of LB broth powder (Sigma, UK L3022) before autoclaving at 121°C for 15 minutes. BHI broth was cooled before use and stored at room temperature.

2. Bacterial strain and inoculum preparation

E. coli RS218, a K1 strain isolated from a case of neonatal meningitis was used for all *E. coli* infection models. This strain was gifted by Dr Stephen Smith, Trinity College, Dublin.

Infections were carried out with mid-log cultures. Strains were streaked on to LB agar plates from frozen stocks and incubated at 37°C for 16-18 hours. The following day universal tubes containing 5 ml of LB broth were inoculated from the plate and incubated at 37°C, 200 rpm for 16-18 hours. Following the 16-18 hour incubation 500 µl was subcultured into 20 ml of LB broth and adjusted to an OD_{600} of 0.1. The culture was incubated at for 37°C, 200 rpm for 1-2 hours until reaching an OD_{600} of 0.5, cultures were

centrifuged at 3000 rpm for 5 minutes, the supernatant discarded and the pellet resuspended in PBS, this was repeated and the culture then resuspended at 2 x 10^8 CFU per ml. Doses were plated onto LB agar to confirm inoculum CFUs .

3. Animal model techniques

All animal work was completed at the University of Liverpool under Home Office project licence No.40/3602. Female CD-1 mice aged between 6-7 weeks were used for all experiments.

a) Euthanasia and animal monitoring

During experiments animal behaviour was monitored to assess progression of infection and wellbeing of animals, scoring system is described in Table 10. In accordance with the home office licence under which experiments were performed, mice were culled when they reached lethargy ++.

b) Schedule 1 culls

Schedule 1 culls were carried out on animals during survival studies. Mice were culled by cervical dislocation or alternatively mice were place in an anaesthetics box and the CO_2 concentration increased slowly over a 6 minute period until mice stopped breathing and cervical dislocation was performed to confirm death.

Table 10 Animal model scoring

Score	Description
Normal	Mouse is displaying normal behaviour and coat condition.
Hunched +	Mouse displays slight arching of back.
Hunched ++	Mouse displays very arched back
Starry +	Coat looks poorly groomed around neck area with upright hairs.
Starry ++	Coat looks poorly groomed over whole animal.
Lethargy +	Mouse is moving slowly around cage.
Lethargy ++	Mouse does not move without encouragement.
Moribund	Mouse is poorly groomed, not moving and has laboured breathing.

c) Cardiac puncture under terminal anaesthesia

Mice were placed in an anaesthetics box and with oxygen set to 0.8 L/min and isofluorane at 5%, mice were monitored, an absence of reflex responses, whisker twitching and a decreased respiratory rate confirmed anaesthesia. Mice were transferred to an anaesthesia cone and the paws and tail firmly pinched to ensure a lack of pain response. A 23 gauge needle attached to a syringe was inserted under the rib cage into the heart at a 45° angle and the syringe pulled back until 1-2 ml of blood is collect. Mice were then immediately culled by cervical dislocation.

d) Animal procedures

(i) Intraperitoneal injection

Intraperitoneal injections were used for the infection of animals during the *E coli* infection models and for administration of IVIG in all animal studies. Mice were manually restrained: held by the scruff with the thumb and forefinger non-dominant hand, securing tail with the remaining fingers. Mice were then tilted downward at a 45 ° angle and injected in either the left or right the lower quadrant of the abdomen, away from the midline to avoid puncturing the bladder with a 27 gauge insulin syringe.

(ii) Intravenous injection

Mice were warmed for 5-10 minutes prior to injection in a heating box to dilate the veins. Mice were secured in a restraint device and the tail held with slight traction and a 27 gauge needle inserted parallel to the vein (either of the lateral veins shown in Figure 21) and advanced 2-3 mm into the lumen before slowly injecting. If the infection is performed correctly the vein should blanche as the substance is injected.

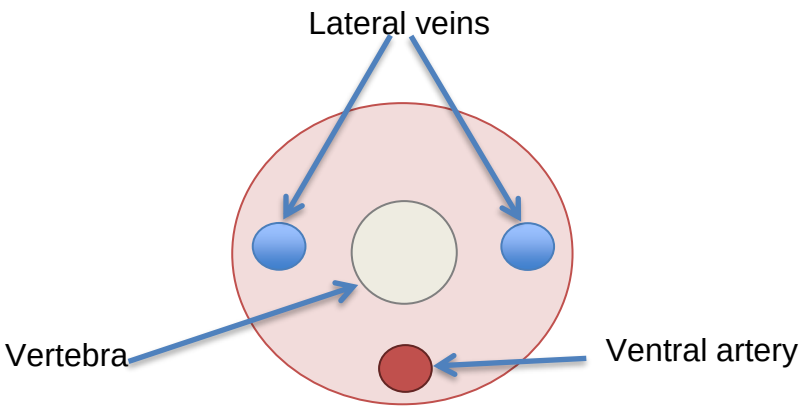


Figure 21 Anatomy of murine tail

e) Infection and monitoring

The prepared dose was administered by intraperitoneal injection into mice as a 50 µl dose, using a 27g insulin syringe (detailed in methods and materials chapter). Because of the extremely rapid onset of disease symptoms mice were monitored hourly from administration of infectious dose. Animals were culled when they reached ++ lethargy (details of scoring in methods and materials).

f) P4 treatment

P4 peptide and IVIG were administered at one and three hours post infection. P4 peptide was administered via a 50 µl intravenous injection into the tail vein at a concentration of 2 mg/ml dissolved in DEPC treated water. This dose was chosen as it was what had been previously used successfully in the treatment of *S. pneumoniae* sepsis (167). IVIG was administered by a 100 µl intraperitoneal injection (I.P.) of Gamunex-C (Grifols, Spain), 100 mg of protein per ml. IVIG was injected I.P. as trials carried out at the CDC showed that the mice better tolerated it when compared with intravenous injection of of IVIG. Control animals received an injection of equivalent volume of sterile PBS.

g) Antibiotic treatment

Tazocin (Pfizer, USA) – piperacillin/tazobactam – was used in experiments at a dose of 9 mg/mouse, which equated to ¼ of the human equivalent dose. Tazocin was injected via the tail vein, 50 µl dissolved in DEPC treated water at the same time points as P4 treatment. Control animals received an

injection of equivalent volume of sterile PBS. Tazocin was chosen as it is a broad-spectrum antibiotic, which is commonly used for peritonitis and pneumonia.

4. Tissue collection

(a) CFUs

Tissue being processed for the determination of CFUs only was homogenised with a T10 homogeniser (IKA, Germany) in 3 ml of PBS, short 5 second bursts at a high speed were used until the tissue suspension appeared homogeneous. The Miles and Misra technique was used to determine CFU numbers from mouse tissues. Using a 96 well plate, 20 µl of bacterial suspension was serially diluted into wells each containing 180 µl of phosphate buffer solution (PBS). Each well was thoroughly mixed before transferring 20 µl to the next dilution; a sterile pipette tip was used for each of the dilutions. Dilutions were continued from 10^1 to 10^6. Agar plates were marked into six sections and labelled from 10 to 10^6, 20 µl of each dilution was plated in triplicate onto the corresponding segment. Plates were then incubated overnight at 37°C. Colonies were counted in sections containing between 30-300 colonies. Colony forming units (CFU) from the original samples were calculated using the following equation:

$$\mathrm{CFU/ml} = \text{Number of colonies in sector} \times \text{dilution factor} \times (1000/60)$$

b) Flow cytometry

Tissue being processed for flow cytometry was placed in a 40 µm cell strainer on top of a 50 ml tube and forced through the membrane with the

plunger from a 2 ml syringe. Cell strainers were rinsed through with 5 ml of PBS. Cells were pelleted by centrifuging at 300 g and the supernatant discarded (or stored at -80 °C for later cytokine analysis). Red blood cells were lysed using red blood cell lysis buffer (eBioscience, UK) according to the manufacturers instructions. Cells were centrifuged at 300 g, the supernatant discarded and the cells resuspended in PBS, 2 % FBS ready for staining for flow cytometry.

Single cell suspensions were divided equally into the wells of a 96 round bottom plate. For each sample there was an unstained well, isotype control well and a stained well. Plates were centrifuged at 300 g and the supernatant removed. The antibodies detailed in Table 11 with corresponding isotype controls (BIolegend, USA), were used for flow cytometry analysis of mouse leukocytes. All antibodies were used at a dilution of 1:250 in staining buffer (PBS supplemented with 2% FBS).

Table 11 Mouse flow cytometry antibodies

Biolegend Product Code	Description
101325	APC anti-mouse CD16/32
101245	Brilliant Violet 510™ anti-mouse/human CD11b
135809	PE/Cy7 anti-mouse CD88 (C5aR)
123417	APC/Cy7 anti-mouse CD21/CD35 (CR2/CR1)
103127	Alexa Fluor® 700 anti-mouse CD45
139303	PE anti-mouse CD64 (FcγRI)
108405	FITC anti-mouse Ly-6G/Ly-6C (Gr-1)

Each well then had 50 µl of stain (or staining buffer for unstained controls) added and the wells were mixed. Cells were incubated for 20 mins at 4°C. Cells were then washed 3 times in staining buffer by centrifuging at 300 g, discarding supernatant and resuspending in 200 µl of staining buffer. Cells were then either immediately analysed by flow cytometry or were fixed with Cytofix (BD, UK) according to the manufacturers instructions, for analysis the following day. Sample acquisition was performed on the BD LSR II (BD Biosciences, USA) and analysis was performed with FlowJo 8.7 (Tree Star, USA).

c) Cytokine analysis

ELISA assays for CXCL1, IL-10 and C5a (R&D systems, UK) were performed according to the manufacturers instructions. Blood was collect in heparin and nafamostat mesylate (Sigma, UK) to prevent complement activation.

5. Statistical analysis

Statistical analysis was performed with Prism 5 (Graphpad Software, USA), individual tests are noted in figure legends.

C. Model development

The mouse model of *E. coli* infection developed for this project, involved injection of *E. coli* RS218 into the peritoneal space of mice. Various doses were trialled during the establishment of this model.

Figure 22 shows the CFUs detectable in the blood of CD-1 mice infected at various doses. The dose of 1 x 10^7 CFU/mouse was chosen as lower infectious doses were clear by the mice within 48 hours without treatment.

Infection with 1 x 10^7 CFUs progressed rapidly, with CFUs detectable in the blood at 3 hours post infection (Figure 22). Untreated mice progressed to lethargy quickly with subjects being culled from eight hours post infection. Other mouse strains were trialled to see if the model could be extended beyond 8 hour survival for untreated animals. No difference was observed in survival times between CD-1 mice and Balb/C, C57B6 or MF1 mouse strains.

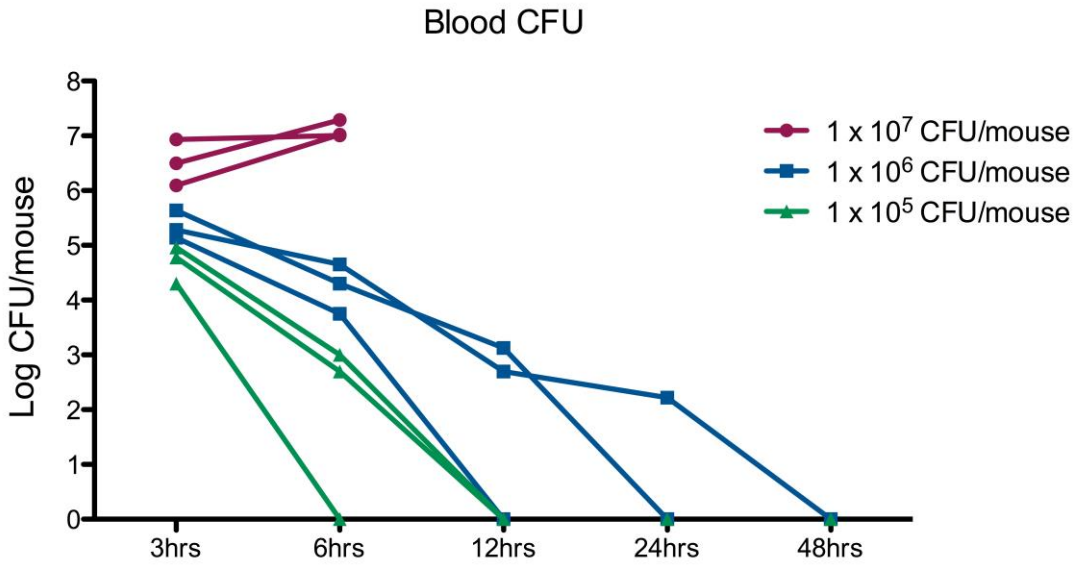


Figure 22 Blood CFUs of mice infected with *E. coli* over 48 hrs
Blood CFUS of mice (n=3 per group) infected with *E. coli* via I.P. injection at dose between 1 x 10^5 and 1 x 10^7 CFU/mouse. Blood was collected from the tail vein at intervals over a 48 hr period and CFUs determined by Miles and Misra.

D. Results

1. Single dose P4 treatment

In order to assess the viability of P4 as a treatment for *E. coli* infection I carried out preliminary experiments aimed at determining if a P4-IVIG treatment could reduce the bacterial burden in the blood, lungs and spleen of mice. Mice were infected with 10^7 CFUs of *E. coli* via I.P. injection followed by a single treatment of P4 (50 µl of 2 mg/ml I.V.) and IVIG (100 µl of 100 mg/ml I.P.) at 1 hour post infection. Experiments tracking bacterial burden in the blood during the model development showed that by 3 hours post infection mice already had between 10^6-10^7 CFU/ml of blood and reached the humane endpoint within 8-12 hours (Figure 22); as the model was known to progress quickly, the earliest logistically possible time point, 1 hour post infection, was chosen for treatment. Animals were then culled at four and 7 hours post infection in order to collect the blood, lungs and spleen and determine the CFUs present. The hours post infection cull was chosen based on the model development data, which showed dissemination of the infection into the blood by 3 hours post infection. The 7 hour post infection cull was chosen as the last time point at which there was 100% survival of untreated animals during model development. Treatment doses of P4 and IVIG were based on those used to successfully treat mice in models of *S. pneumoniae* sepsis (167).

Treatment with a single dose of P4 and IVIG led to substantial decreases (1 log) in CFUs detected in the blood, lungs and spleen at both the four and 7 hour time points (Figure 23 and Figure 24), with the seven hour time point

showing a 2 log decrease in CFU burden in the blood and spleen (blood mean PBS = 4.4 x 10^8 CFU/ml vs P4-IVIG = 8.6 x 10^6 CFU/ml, p=0.0004, spleen mean PBS = 4.8 x 10^5 CFU/mg vs P4-IVIG = 3.1 x 10^5 CFU/mg, p=0.02) and a 5 fold decrease in lung CFUs (mean PBS = 5.0 x 10^4 CFU/mg vs P4-IVIG 1.0 x 10^4 CFU/mg, p=0.007).

During the model development it was observed that mice infected with lower doses of *E. coli* (10^6 and 10^5 CFU/mouse) had 10^6 CFU/ml of blood or less at 3 hours post infection and that the bacterial burden would subsequently decline to undetectable levels over 48 hours without intervention; those with CFUs in the blood over 10^6 CFU/ml succumbed to their infection. Although decreases in CFUs were promising, the number of CFUs in the blood and spleen of mice that received P4 treatment was static between the two time points, remaining at above 10^6 CFU/ml in the blood. In addition to this the number of CFUs detected in the lung had increased by close to 1 log between the two time points (mean four hour = 1.3 x 10^3 CFU/mg vs 7 hours = 1.0 x 10^4 CFU/mg). With this in mind I concluded that a single dose regime was unlikely to lead to significant increases in survival and either concurrent treatment with antibiotics or additional treatment doses would be needed.

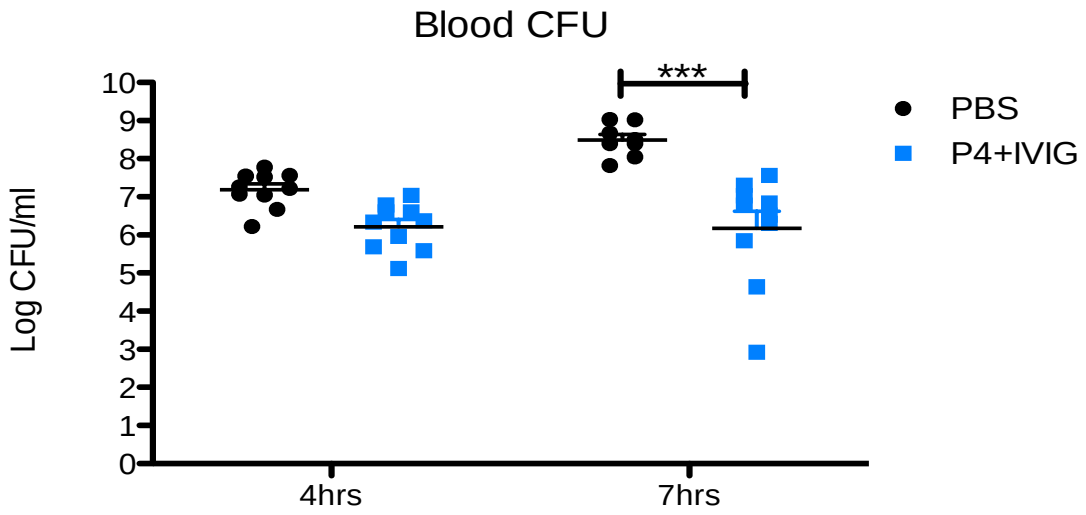


Figure 23 Single dose treatment of P4 peptide in severe *E. coli* infection model – Blood CFUs

Mice n=10 per group were infected with 1 x 10^7 CFU *E. coli* via the intraperitoneal route. 1 hour post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and I.P. with 100 µl of IVIG (100 mg/ml), while control animals received injections of PBS of equal volume. Mice were culled 4 and 7 hours post infection and blood collected for determination of CFUs. Plotted as mean and SEM. Analysis by two-way ANOVA p=0.0004 (*** p<0.0001).

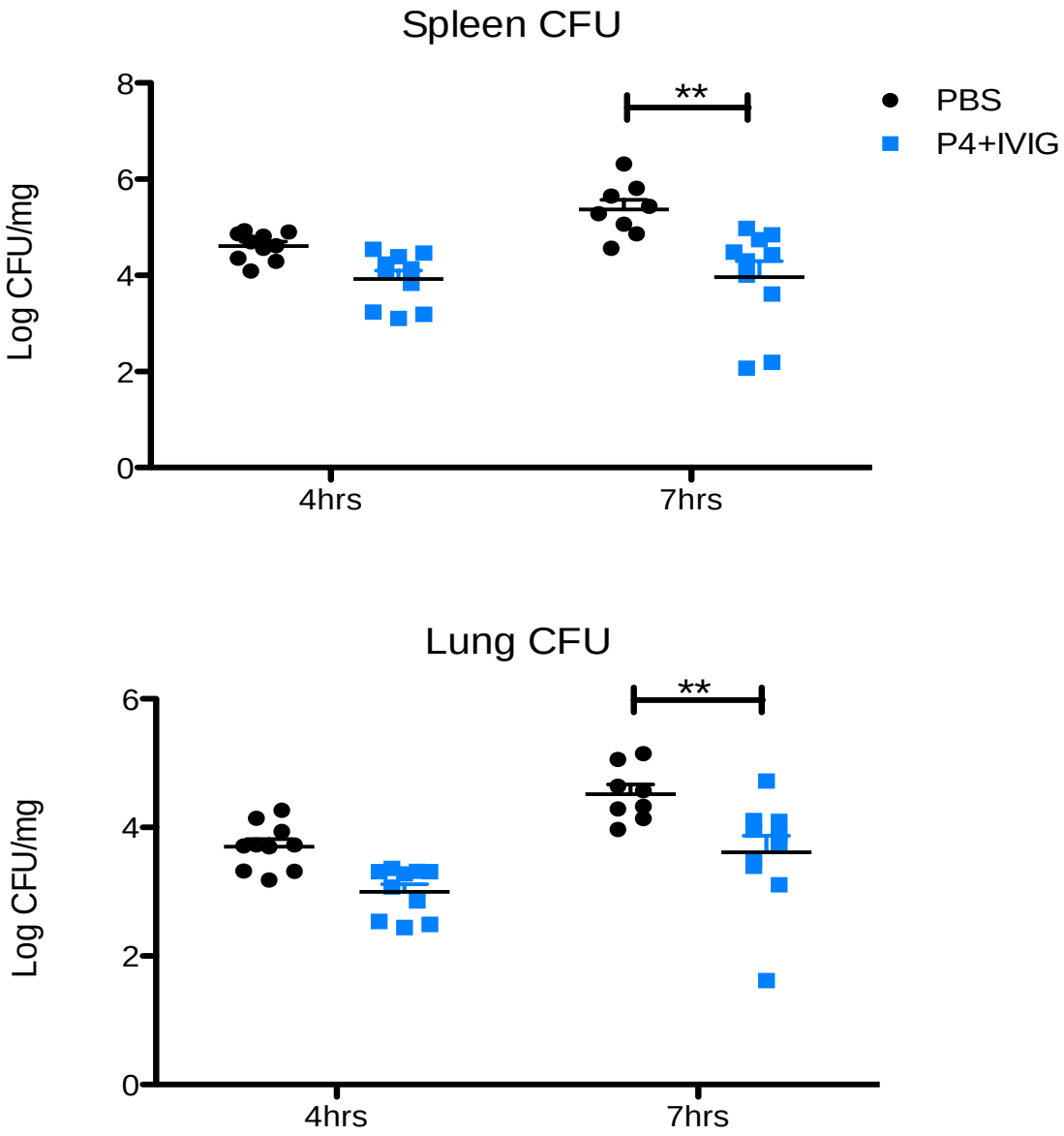


Figure 24 Single dose treatment of P4 peptide in severe *E. coli* infection model – Tissue CFUs

Mice n=10 per group were infected with 1 x 10^7 CFU *E. coli* via the intraperitoneal route. 1 hour post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and I.P. with 100 µl of IVIG (100 mg/ml), while control animals received injections of PBS of equal volume. Mice were culled 4 and 7 hours post infection and tissue collected for determination of CFUs. Plotted as mean and SEM. Analysis by two-way ANOVA, spleen p=0.02, lung p=0.007, ** p<0.005.

2. Single dose combination treatment

a) Tazocin testing

The single dose P4 treatment experiments showed reductions in CFUs detected in the blood, lungs and spleen of P4-IVIG treated mice, however did not show reductions that were likely to lead to an increase in survival. Following these experiments I then went on to looked at P4 treatment in combination with antibiotics. The antibiotic chosen for these experiments was Tazocin; a dose of 9 mg/mouse was selected as ¼ of the human equivalent dose (based on an average mouse weight of 25 g). To test this dose animals were infected with 10^7 CFU of *E. coli* before treating one hour post infection with 9 mg/mouse of Tazocin via I.V. injection, mice were bled at four hours post infection and culled at seven hours post infection to monitor the bacterial burden in the blood.

Treatment with a single dose of Tazocin at 1 hour post infection lead to a reduction in CFUs recovered from blood of over 2 log (mean PBS = $6.8x10^7$ CFU/ml vs Tazocin = $4.3x10^5$ CFU/ml at the four hour time point; PBS = $9.7 x10^8$ CFU/ml vs Tazocin = $1.6 x10^6$ CFU/ml at the seven hour time point, two-way repeated measures ANOVA $p<0.0001$) (Figure 25).

Although this antibiotic concentration lead to a significant reduction in CFUs it did not clear the infection and the numbers of CFUs detected were increasing between the two time points. It was decided to continue with this dose in the combination treatment experiments as it was sufficient to reduce the numbers of CFUs but not so high that it would mask any potential benefit seen with P4 treatment.

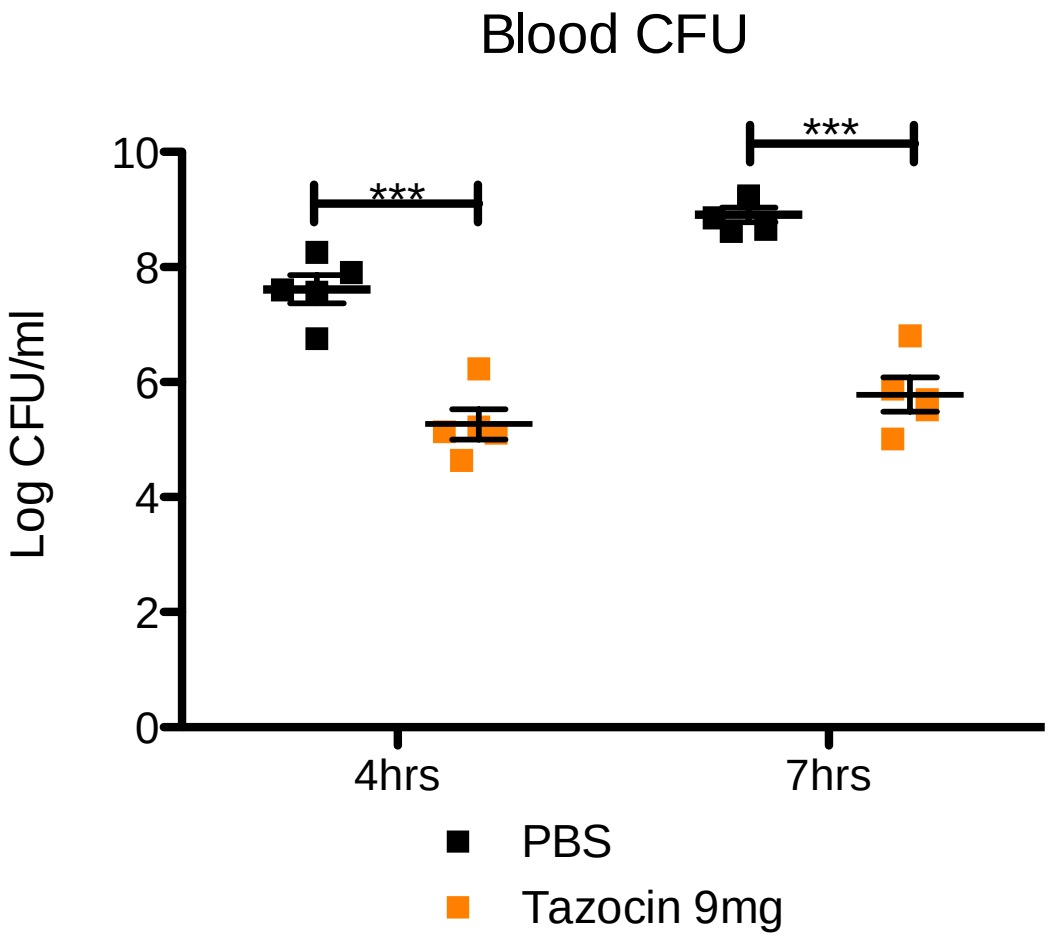


Figure 25 Blood CFU after single treatment of Tazocin in *E. coli* infection model

Mice n=5 per group were infected with 1 x 10^7 CFU *E. coli* via intraperitoneal route. 1 hour post infection mice were treated I.V. with 50 µl of Tazocin (9 mg/mouse) or 50 µl of PBS. Mice were tail bled at four hours post infection and culled at seven hours post infection. Plotted as mean and SEM. Blood was collected for determination of CFUs. Analysis by two-way ANOVA *p*=0.0001 (*** p<0.0001)

b) Bacterial burden and survival

Having established that a single dose of P4 treatment was insufficient to treat the infection and identified a suitable concentration of Tazocin for use in the model I then went on to test the two treatments in combination. Treatment a single dose of P4-IVIG alongside Tazocin was trialled in the *E. coli* mouse model with both agents being administered at one hour post infection; P4 and Tazocin were both administered via I.V. injection whilst the IVIG was administered via I.P. injection. Initial experiments sought to establish the effect of combination treatment on the bacterial burden at four and seven hours post infection as well as survival over a 24 hour period. As in previous experiments, animals were infected with 10^7 CFU of *E. coli* before treating one hour post infection with either PBS, P4-IVIG, Tazocin or the combination of P4-IVIG and Tazocin. Mice were bled at four hours post infection and culled at seven hours post infection to monitor the bacterial burden in the blood and lungs. A second set of mice were monitored over a 24 hour period as a survival experiment, with mice being culled when they reached their humane end point of lethargy.

Treatment with the two drugs in combination appeared to have an additive effect; the decrease in CFUs being approximately the sum of the decrease in CFUs of each of the two treatments individually (Figure 26). There were significantly lowers numbers of blood CFUs detected for all three treatments at seven hours post infection compared with PBS treated mice: P4-IVIG + Tazocin = 1.7 x10^6 CFU/ml (p<0.005), Tazocin = 1.5 x 10^8 CFU/ml (p<0.05), P4-IVIG = 4.5 x 10^7 CFU/ml (p<0.005) and PBS = 5.3 x 10^8 CFU/ml. The four hour time point showed the same trend but no individual pairings were

statistically significant (two-way ANOVA p=0.04). The lung CFUs showed the same trend as the blood CFUs but was not statistically significant (p=0.1, one-way ANOVA) (Figure 27).

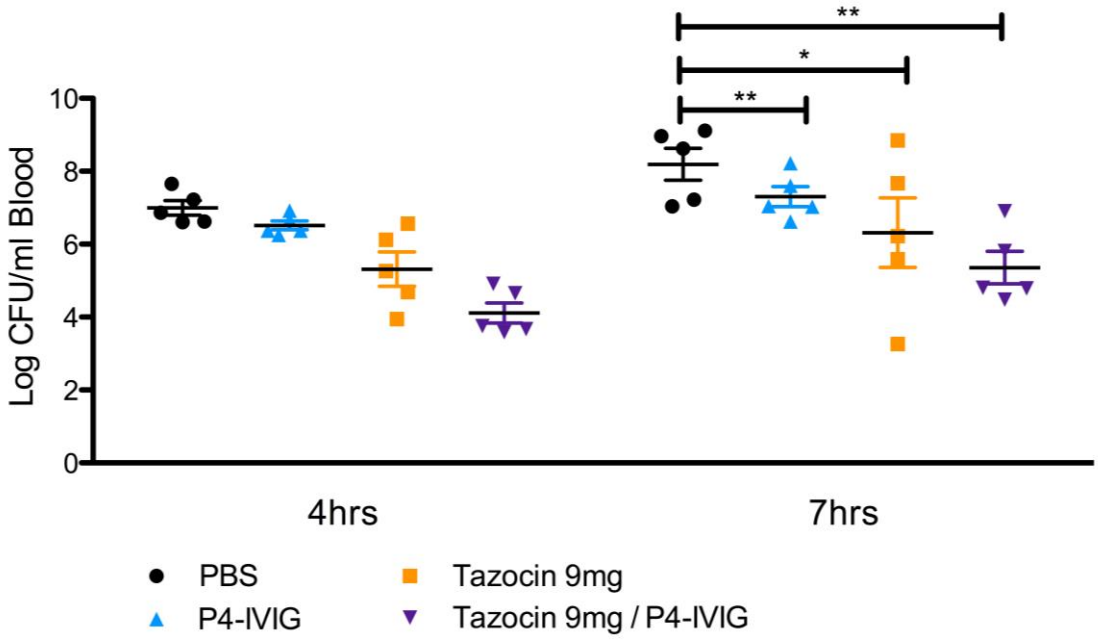


Figure 26 Single dose P4 therapy with antibiotics in *E. coli* infection model – Blood CFU

Mice n=5 per group were infected with 1 x 10^7 CFU *E. coli* via the intraperitoneal route. 1 hour post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were tail bled at 4 hours post infection and culled at 7 hours post infection. Blood was collected for determination of CFUs. Plotted as mean and SEM. Analysis by two-way ANOVA and Bonferroni post tests p=0.0491 (** p<0.005, * p<0.05).

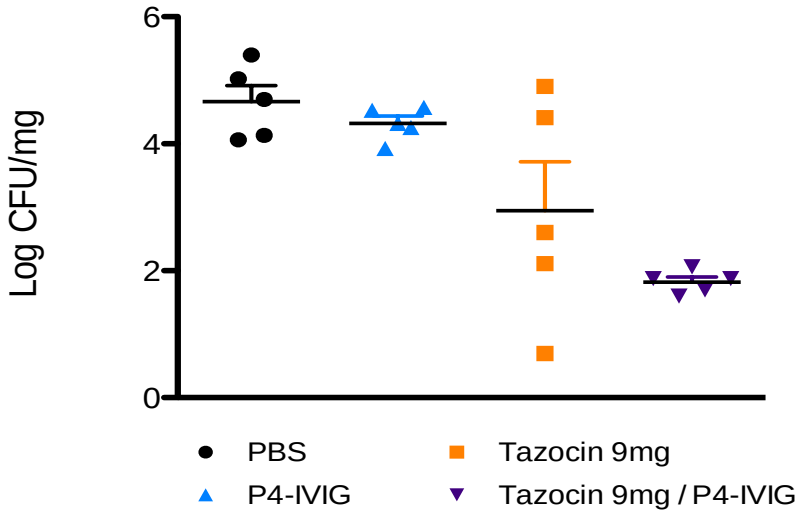


Figure 27 Single dose P4 therapy with antibiotics in *E. coli* infection model – Lung CFU 7 hours post infection

Mice n=5 per group were infected with 1 x 10^7 CFU *E. coli* via the intraperitoneal route. 1 hour post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were culled at seven hours post infection. Lung tissue was collected for determination of CFUs. Plotted as mean and SEM. Analysed by one-way ANOVA and Bonferroni's multiple comparison test *p*=0.1

In the survival model mice were monitored over a 24 hour period. The mean survival time was significantly higher for mice treated with either of the three treatment groups when compared to the untreated (PBS) control: Tazocin alone 15.8 hours (p<0.005), P4-IVIG alone 16.8 hours (p<0.005) and P4-IVIG + Tazocin 19 hours (p<0.0001) compared with PBS treated mice 8.4 hours (one-way ANOVA and Bonferroni's multiple comparison test, p=0.0004). There was no difference in mean survival between the three treatment groups (Figure 28, Figure 29 and Table 12).

The survival percentage at the end of the 24 hour period was 40% for the P4-IVIG + Tazocin treatment group but was 0% for all other treatment groups. Blood was collected at the time of death for mice in the survival experiments and the CFUs in blood determined; it was observed that some of the mice treated with Tazocin and P4-IVIG + Tazocin had lower CFUs than you would expect at the time of death (Figure 30). This could suggest that factors such as excessive inflammation or high levels of endotoxins could be contributing to mortality in addition to the bacterial burden.

As survival within the P4+IVIG and Tazocin group was still low, at 40% over 24 hours, I decided to increase the number of doses administered to try and improve survival.

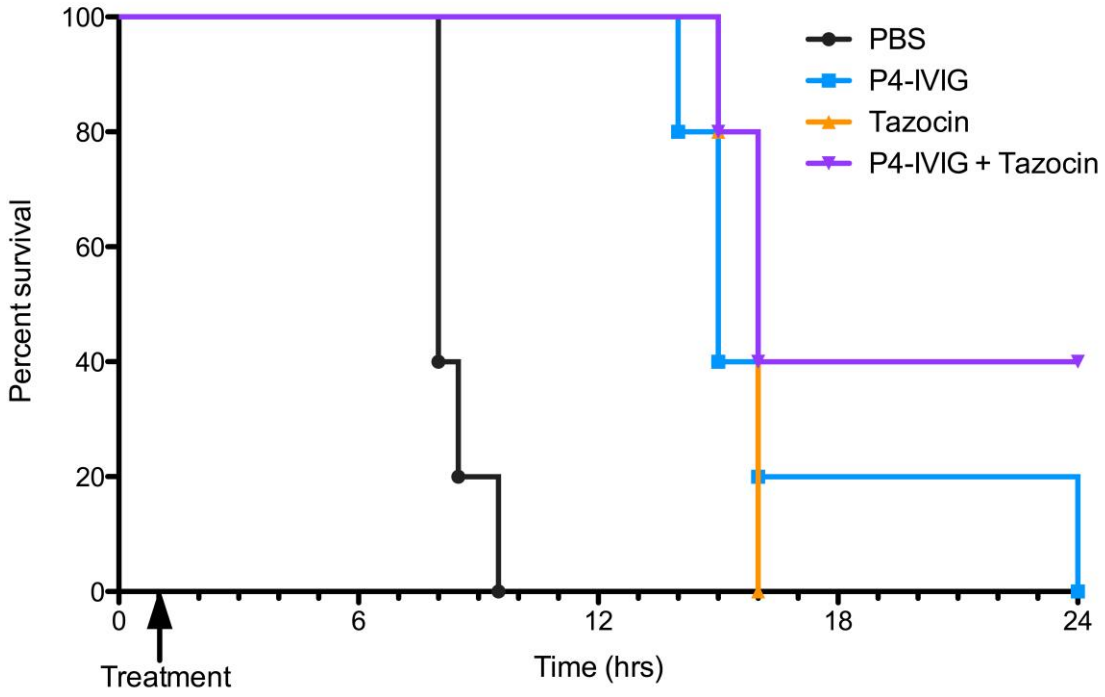


Figure 28 Single dose P4 therapy with antibiotics in *E. coli* infection model - Survival
Mice n=5 per group were infected with 1 x 10^7 CFU *E. coli* via intraperitoneal infection. 1 hour post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were monitored for signs of disease and culled when they reached ++ lethargic.

Table 12 Single dose P4 therapy with antibiotics in *E.coli* infection model – Survival time

	PBS	Tazocin	P4-IVIG	P4-IVIG + Tazocin
Group size	5	5	5	5
Mean	8.40	16.80	15.80	19.00
Minimum	8.00	14.00	15.00	15.00
Maximum	9.50	24.00	16.00	24.00

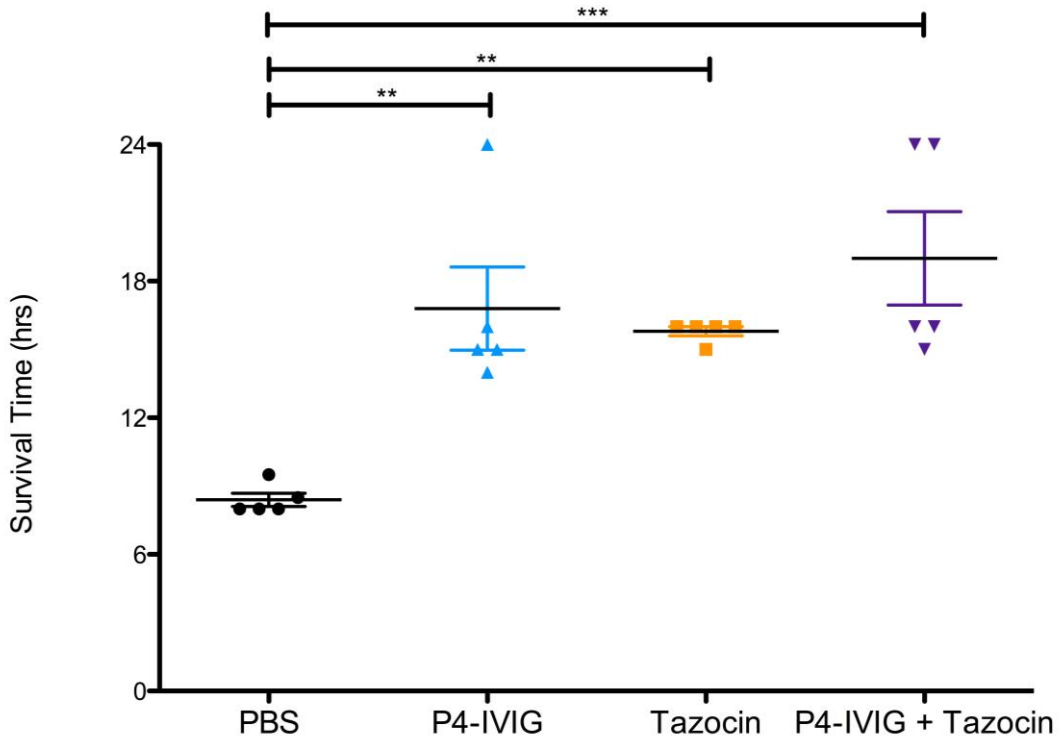


Figure 29 Single dose P4 therapy with antibiotics in *E. coli* infection – Survival time

Mice n=5 per group were infected with 1 x 10^7 CFU *E. coli* via the intraperitoneal route. 1 hour post infection mice were treated I.V. with 50 µl of P4 peptide (1 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were monitored for signs of disease and culled when they reached ++ lethargic. Survival time in hours and the median plotted. Analysed by Kruskal-Wallis test and Dunn's multiple comparison test *** p<0.001, ** p<0.005

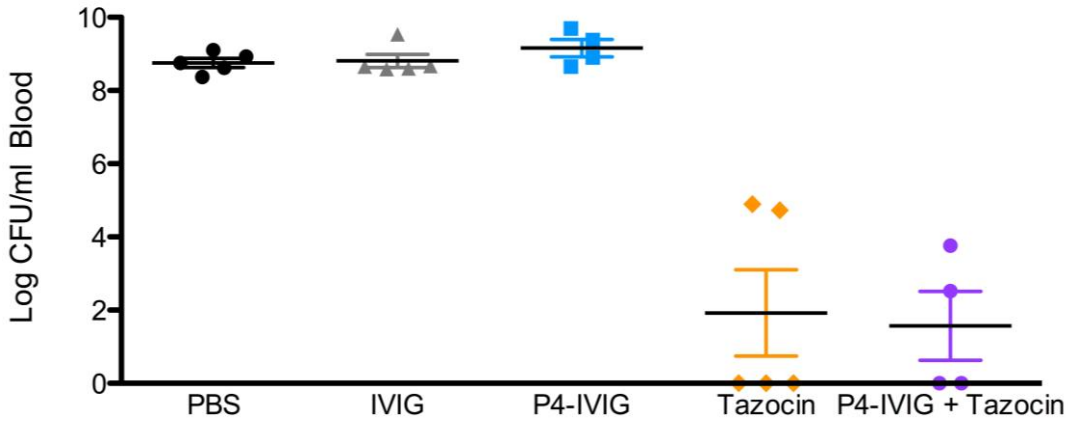


Figure 30 Time of death blood CFUs

Mice n=5 per group were infected with 1 x 10^7 CFU *E. coli* via the intraperitoneal route. 1 hour post infection mice were treated I.V. with 50 µl of P4 peptide (1 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were monitored for signs of disease and culled when they reached ++ lethargic. Blood was collect at time of death and CFUs determined.

3. Double dose P4 and antibiotic combination treatment

a) Bacterial burden and survival

The next treatment schedule tested was a double dose P4 and antibiotic combination. A second dose was added to the treatment schedule to try and improve survival in the model beyond the 40% survival seen with a single dose. For this set of experiments the Tazocin + IVIG and IVIG alone groups were added after observing that mice in the Tazocin alone treatment group which were culled upon reaching ++lethargic often had considerably lower CFUs at time of death than PBS groups or treatments that did not include Tazocin (Figure 30). The reasoning behind this was that if the mice were succumbing to the infection despite clearing significant numbers of bacteria then perhaps they were having a severe inflammatory response which could be mediated by the via the anti-inflammatory properties of IVIG.

(1) Survival

As the single dose experiments (Figure 26 and Figure 27) had shown the combination of P4-IVIG and Tazocin were effective at reducing the bacterial burden the survival experiments were performed first. As with previous experiments mice were infected with 10^7 CFU/mouse of *E. coli*, treatments were administered at one and four hours post infection with P4 and Tazocin administered via I.V. injection and IVIG via I.P. injection. Mice were then monitored over a 24 hour period and culled when they reached their humane end point.

The P4-IVIG + Tazocin group and the Tazocin + IVIG had 24 hour survival of 55% and 60% respectively Survival for PBS, IVIG, Tazocin and P4-IVIG treated mice were 0%, 10%, 11% and 28% respectively (Figure 31). The mean, minimum and maximum survival times for each group are detailed in Table 13. All treatments bar IVIG alone had significantly longer mean survival than PBS controls.. P4-IVIG + Tazocin had significantly longer mean survival times than PBS, IVIG and Tazocin alone ($p<0.0001$). Tazocin + IVIG had significantly longer mean survival than IVIG alone ($p<0.05$) but did not show a significant difference compared to Tazocin alone. There was no significant difference between mean survival times for P4-IVIG + Tazocin and Tazocin + IVIG (Figure 32).

The mean survival time and survival percentage showed improvements with the double dose compared with the single dose; survival percentages over 24 hours increased from 40% to 55% for P4-IVIG and Tazocin and the survival percentage for Tazocin increased from 0% to 60% (Figure 28 and Figure 31). However, mean survival times did not show improvements: P4+IVIG and Tazocin mean survival time single dose = 19 hours vs. double dose = 20.6 hrs, Tazocin mean survival time single dose = 16.8 hours vs. double dose = 13.8 hours (Table 12 and Table 13).

As there was no significant difference between the survival percentages or mean survival times of mice treated with P4-IVIG and Tazocin versus those treated with Tazocin and IVIG I went on to look at the bacterial load and inflammatory markers.

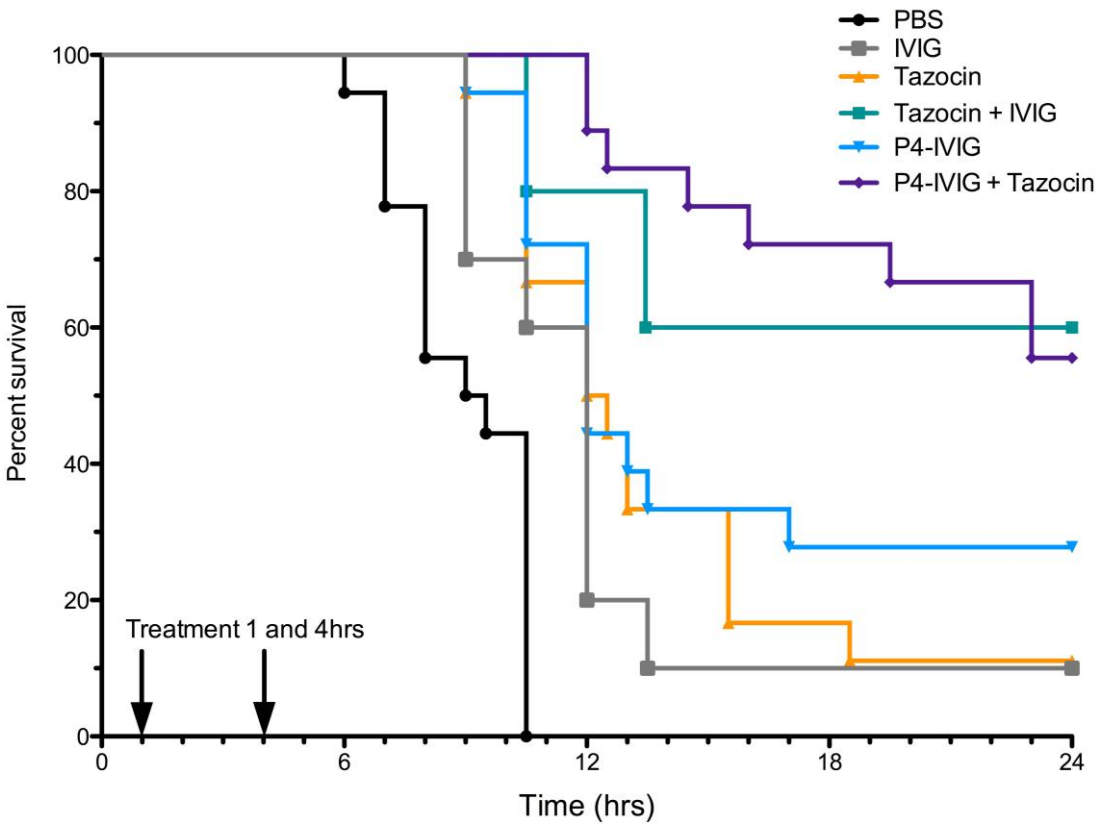


Figure 31 Double dose P4 therapy with antibiotics in *E. coli* infection model – Survival

Mice n=18 (except IVIG and Tazocin + IVIG n=10) per group were infected with 1 x 10^7 CFU *E. coli* via the intraperitoneal route. 1 and 4 hours post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were monitored for signs of disease and culled when they reached ++ lethargic.

Table 13 Double dose P4 therapy with antibiotics in *E. coli* infection model – Survival time

	PBS	IVIG	Tazocin	Tazocin + IVIG	P4-IVIG	P4-IVIG + Tazocin
Group size	18	10	18	10	18	18
Mean	8.972	12.30	13.83	19.19	15.25	20.69
Minimum	6.000	9.000	9.000	10.50	9.000	12.00
Maximum	10.50	24.00	24.00	24.00	24.00	24.00

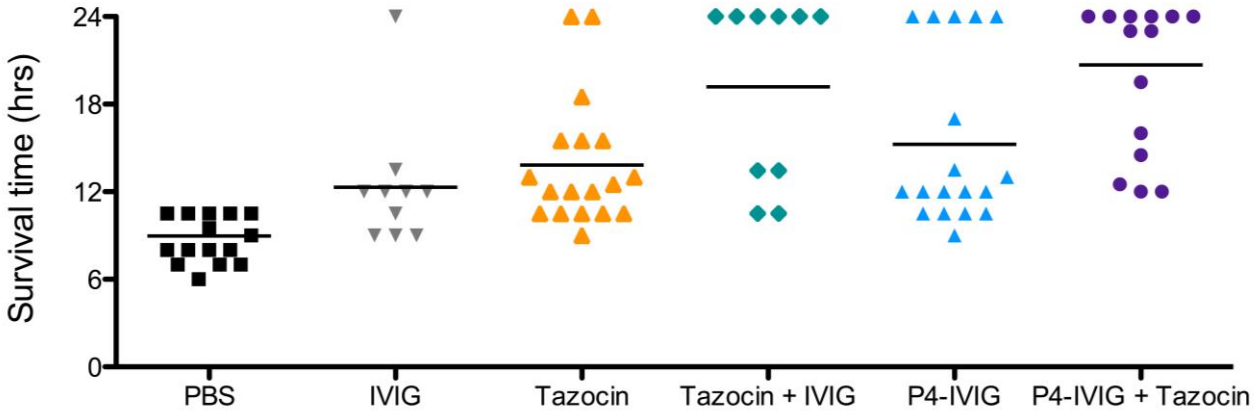


Figure 32 Double dose P4 therapy with antibiotics in *E. coli* infection model – Survival time

Mice n=18 (except Tazocin + IVIG n=10) per group were infected with 1 x 10^7 CFU *E. coli* via the intraperitoneal route. 1 and 4 hours post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were monitored for signs of disease and culled when they reached ++ lethargic. Survival time in hours and the mean plotted. Analysed by one-way ANOVA test, $p<0.0001$ and Bonferroni's multiple comparison test – right hand table (*** p<0.0001, ** p<0.005, * p<0.05)

Pairing	Significance
PBS vs IVIG	ns
PBS vs Tazocin	*
PBS vs P4-IVIG	**
PBS vs P4-IVIG + Tazocin	***
PBS vs Tazocin + IVIG	***
IVIG vs Tazocin	ns
IVIG vs P4-IVIG	ns
IVIG vs P4-IVIG + Tazocin	***
IVIG vs Tazocin + IVIG	*
Tazocin vs P4-IVIG	ns
Tazocin vs P4-IVIG + Tazocin	***
Tazocin vs Tazocin + IVIG	ns
P4-IVIG vs P4-IVIG + Tazocin	*
P4-IVIG vs Tazocin + IVIG	ns
P4-IVIG + Tazocin vs Tazocin + IVIG	ns

(2) Bacterial burden

Having seen an improvement in survival percentages for mice treated with a double dose of treatment but not an improvement in mean survival time I then went on to look at the bacterial burden. Mice were infected with 10^7 CFU/mouse of *E. coli* via I.P. injection; mice were then treated at one hour and four hours post infection with P4 and Tazocin administered via I.V. injection and IVIG via I.P. injection. At seven hours post infection mice were culled and Blood, lungs and spleen tissue were collected and processed for the determination of CFUs. IVIG alone was not included as a group as it showed no benefit over PBS treated mice in survival studies.

(a) Blood CFUs

All treatments containing Tazocin showed a statistically significant reduction in CFUs compared to PBS controls (*p* value of specific comparisons as determined by Dunn's multiple comparison indicated in Figure 33). Differences between the three groups containing Tazocin; Tazocin alone, Tazocin-IVIG and P4-IVIG + Tazocin were not statistically significant although the differences observed in blood CFUs were far larger than in other tissues, with Tazocin + IVIG showing far superior bacterial clearance; Tazocin only median = 8.2×10^4, IQR 3.1×10^3- 2.9×10^5, Tazocin-IVIG median = 0, IQR 0-12, P4-IVIG + Tazocin median = 1.9×10^3 , IQR 77 – 1.5×10^4 (Figure 33).

(b) Lung CFUs

As was seen in the blood, all treatments containing Tazocin showed a statistically significant reduction in CFUs when compared to PBS controls and P4-IVIG (*p* value of specific comparisons as determined by Bonferroni's post test indicated in Figure 34). Differences between the three groups containing Tazocin; Tazocin alone, Tazocin + IVIG and P4-IVIG + Tazocin were not statistically significant. The numbers of CFUs detected in the lungs were far closer for the Tazocin containing groups than was seen in the blood with the medians for the three groups ranging from 10 to 30 CFU/ml (Figure 34). The number of CFU recovered from the lungs of P4-IVIG treated mice did not differ greatly between single and double dose treated mice with a slight increase in the double dose (mean log CFU single dose = 4.3 vs. double dose = 5.2); this could suggest that treatment with P4-IVIG was not as effective it penetrating tissues as antibiotics.

As the bacterial burden was significantly reduced with a double dose of treatment when compared to a single dose of treatment and increased the percentage of survivors over 24 hours, but did not significantly alter the mean survival time of subjects. In an attempt to explain the discordance between these three measures I then went on to look at inflammatory markers, cell populations and endotoxins.

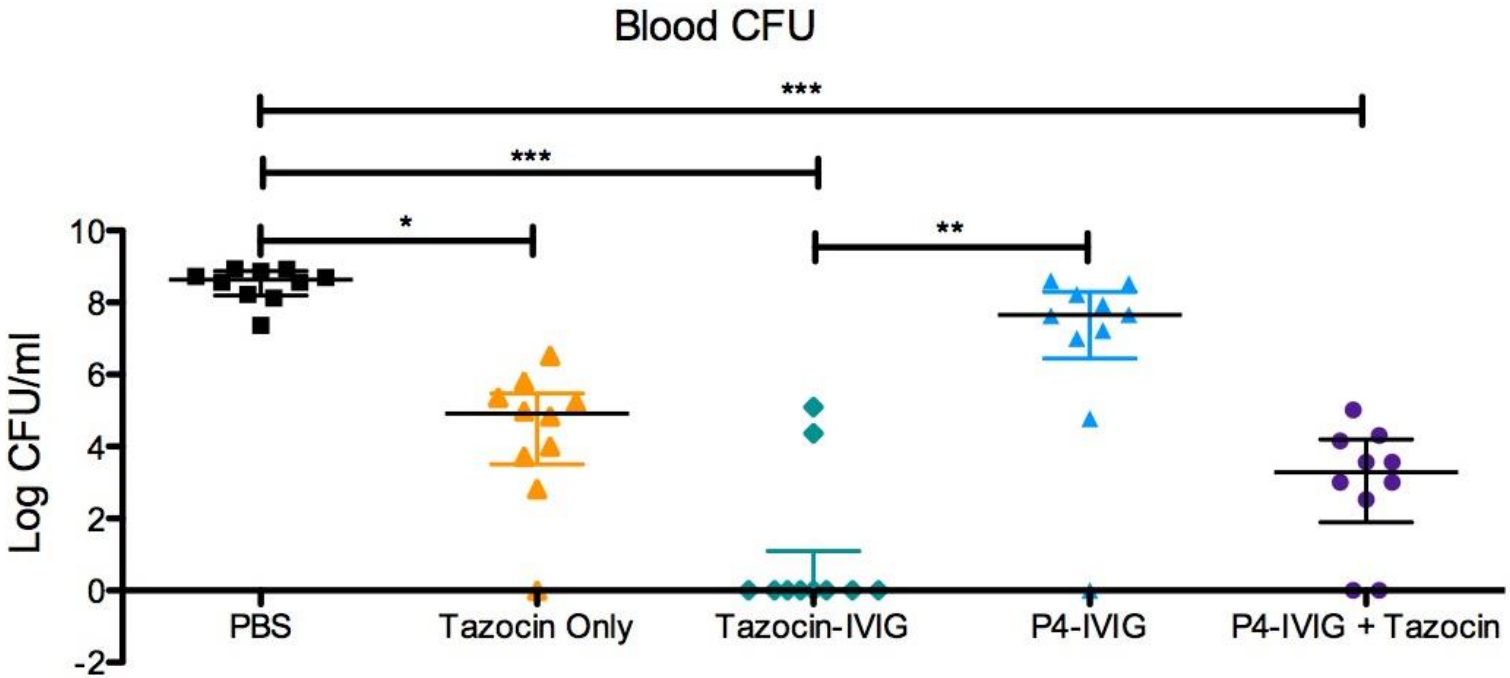


Figure 33 Double dose P4 therapy with antibiotics in *E. coli* infection model –Blood CFU
Mice n=18 (except IVIG and Tazocin + IVIG n=10) per group were infected with 1 x 10^7 CFU *E. coli* via the intraperitoneal route. 1 and 4 hours post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were culled at 7 hours post infection and tissue collected for determination of CFUs. Analysed by Kruskal-Wallis test and Dunn's multiple comparison test with median and interquartile range (*** $p<0.0001$, ** $p<0.005$, * $p<0.05$).

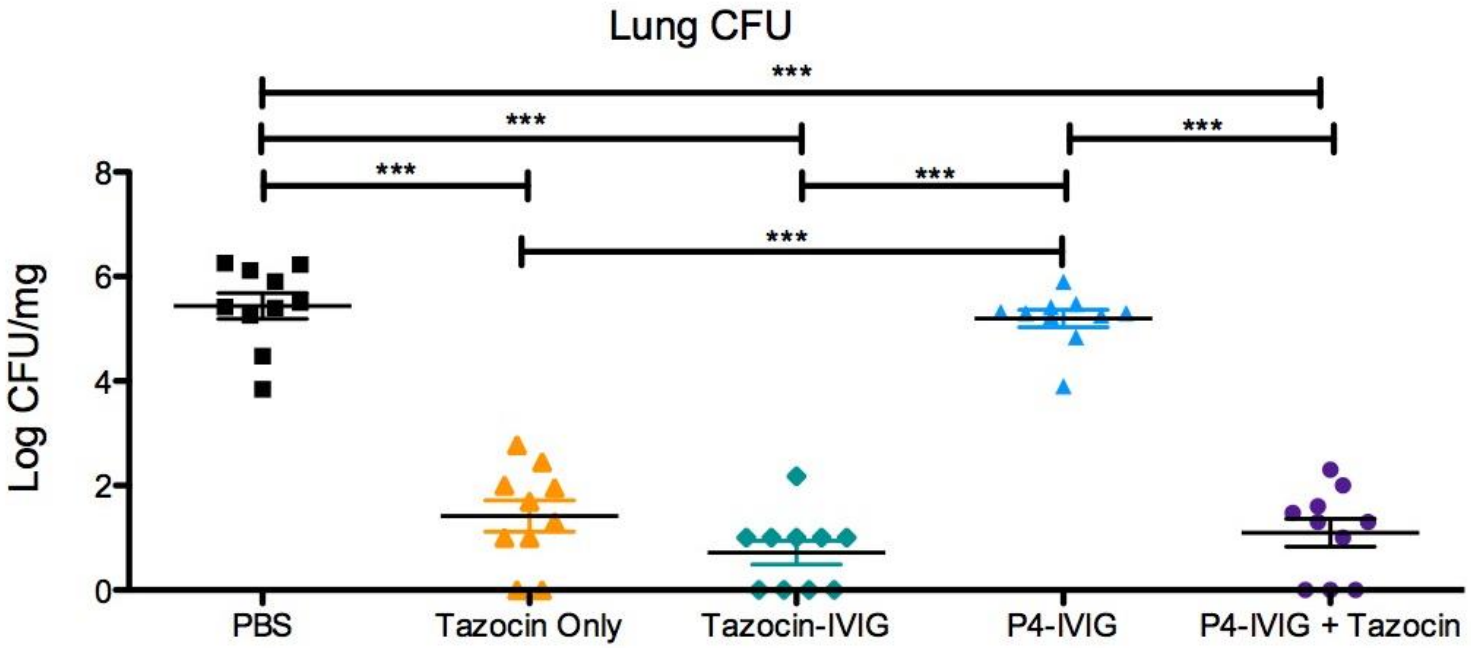


Figure 34 Double dose P4 therapy with antibiotics in *E. coli* infection model – Lung CFU
Mice n=18 (except IVIG and Tazocin + IVIG n=10) per group were infected with 1 x 10^7 CFU *E. coli* via the intraperitoneal route. 1 and 4 hours post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were culled at 7 hours post infection and tissue collected for determination of CFUs. Analysed by one-way ANOVA and Bonferroni post test with mean and SEM *** $p<0.0001$

b) Inflammatory markers

I went on to look at the plasma levels of CXCL1, IL-10 and C5a in mice that had been infected with *E. coli* and treated with a double dose of P4-IVIG and Tazocin. I looked at the time point of seven hours post infection in line with the time point used for assessing bacterial burden. There were two aims to these experiments: looking to see if the different treatment combinations resulted in differences in inflammatory marker levels and to see if the levels of these markers gave any clue as to why mice that had low levels of CFUs reached their humane endpoint and had to be culled.

As with previous experiments mice were infected with 10^7 CFU/mouse of *E. coli* and treated one and four hours post infection before culling at seven hours post infection for blood collection.

Significantly lower levels of CXCL1 ($p<0.005$), were detected in mice treated with P4-IVIG + Tazocin compared with Tazocin alone (Mean P4-IVIG + Tazocin = 181.2 ng/ml vs. Tazocin 824.0 ng/ml, (Figure 35). It should be noted however that all the CXCL1 levels were very high.

The levels of IL-10 detected in plasma were lower in all mice with treatments containing IVIG although these differences were only significant between PBS and Tazocin only treated mice, which had the highest levels (mean IL-10 PBS = 609 pg/ml, Tazocin = 499 pg/ml) and P4-IVIG and Tazocin-IVIG treated mice which had the lowest levels (mean IL-10 Tazocin-IVIG = 90 pg/ml, P4-IVIG = 57 pg/ml) (Figure 36).

There was no difference between groups in the level of C5a detected in plasma (Figure 37). These samples were treated with nafamostat mesylate (FUT-175, Sigma, UK) a serine protease inhibitor that prevents the activation of C5 and production of C5a *ex vivo,* however it is still possible that *ex vivo* activation has occurred that would effect the reliability of this measurement (175). C5a and its corresponding receptors are rapidly internalised upon binding so a lack of difference in plasma concentrations could also be a result of difference in consumption rate between groups (130).

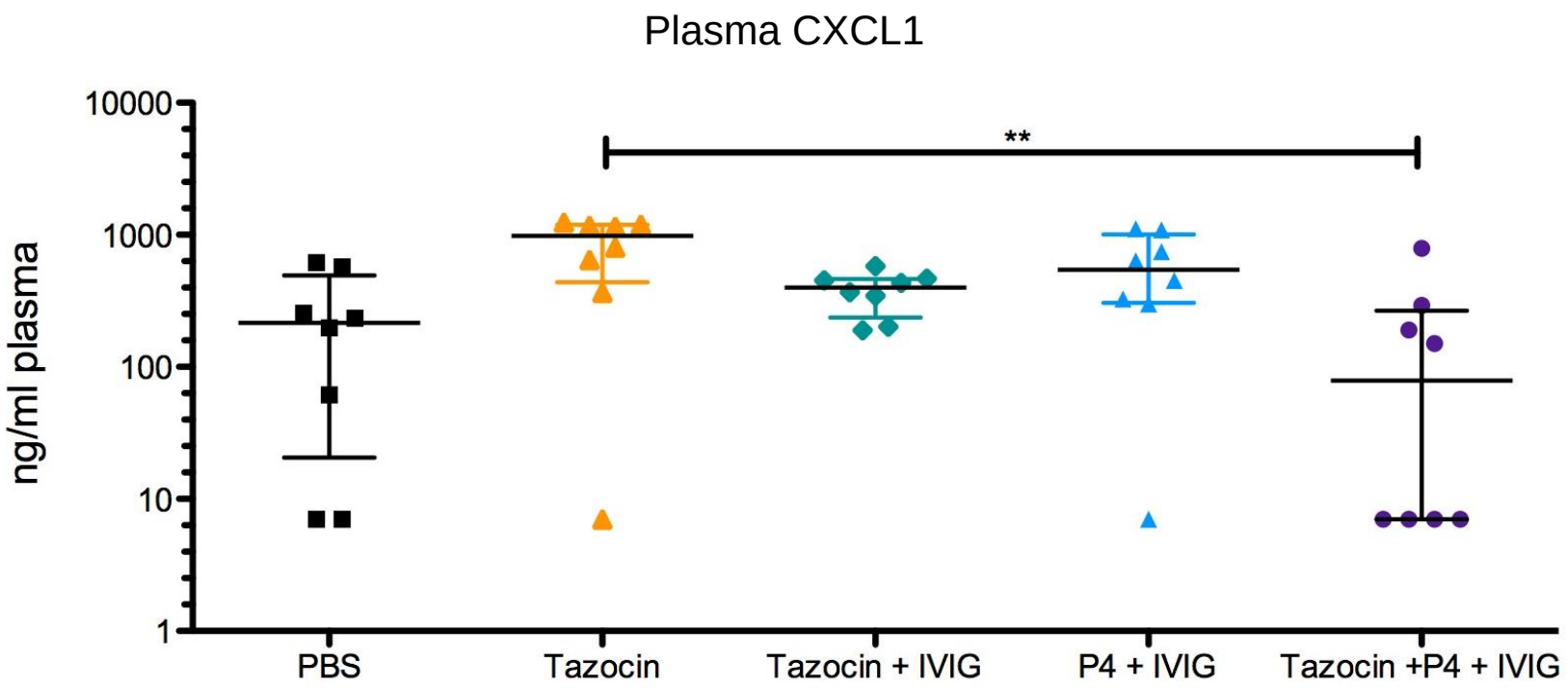


Figure 35 Double dose P4 therapy with antibiotics in *E. coli* infection model – Plasma CXCL1
Mice n=8 per group were infected with 1 x 10^7 CFU *E. coli* via the intraperitoneal route. 1 and 4 hours post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were culled at seven hours post infection and blood collected for determination of cytokine concentrations in plasma. Plotted as mean and SEM. Analysed by Kruskal-Wallis test and Dunn's multiple comparison test p=0.0068 ** p<0.005

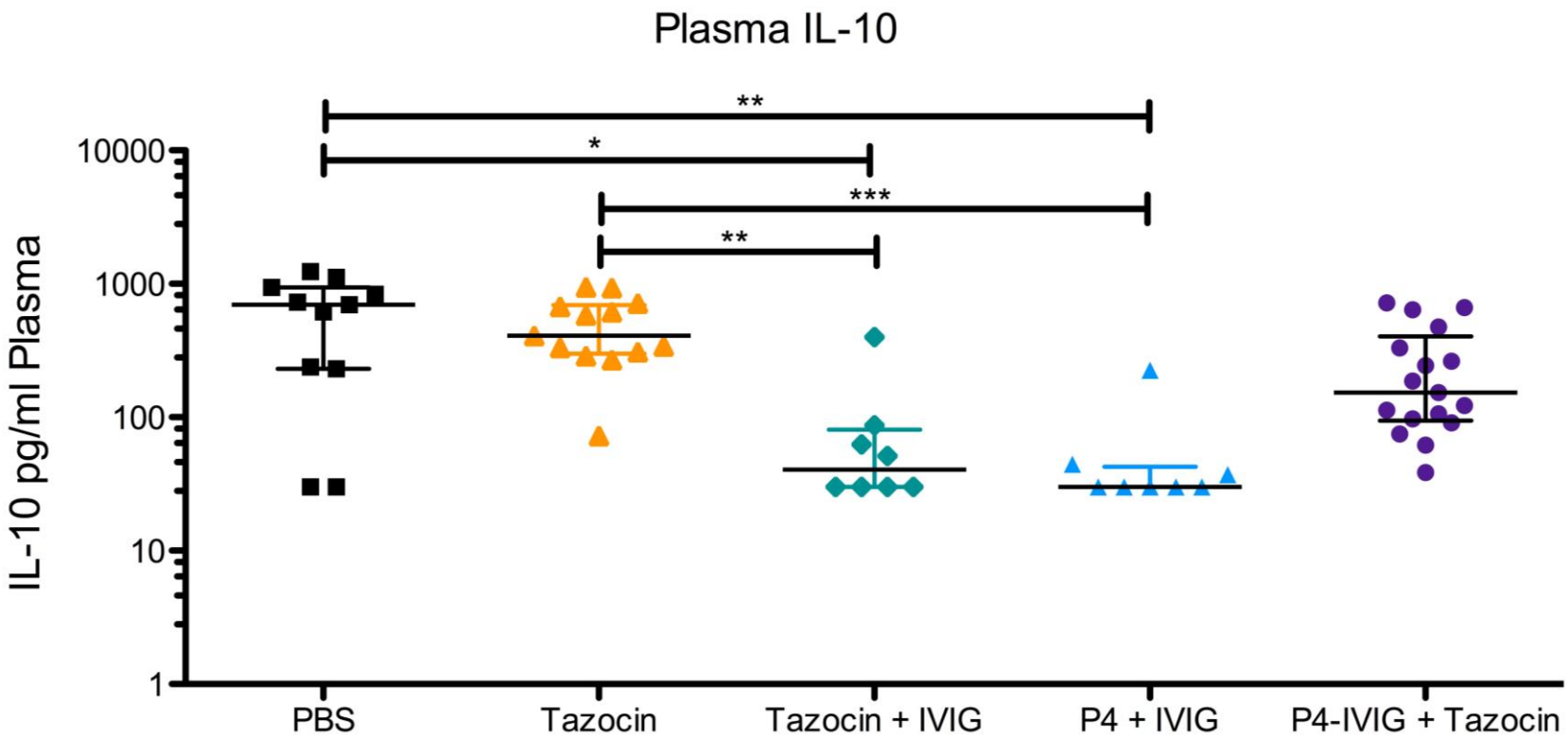


Figure 36 Double dose P4 therapy with antibiotics in *E. coli* infection model – Plasma IL-10
Mice (PBS =11, Tazocin= 13, Tazocin+IVIG=8, P4-IVIG=8, P4-IVIG+Tazocin=17 per group) were infected with 1 x 10^7 CFU *E. coli* via the intraperitoneal route. 1 and 4 hours post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were culled at seven hours post infection and blood collected for determination of cytokine concentrations in plasma. Plotted as mean and SEM. Analysed by Kruskal-Wallis test and Dunn's multiple comparison test p=0.0008 (*** p<0.0001, ** p<0.005, * p<0.05)

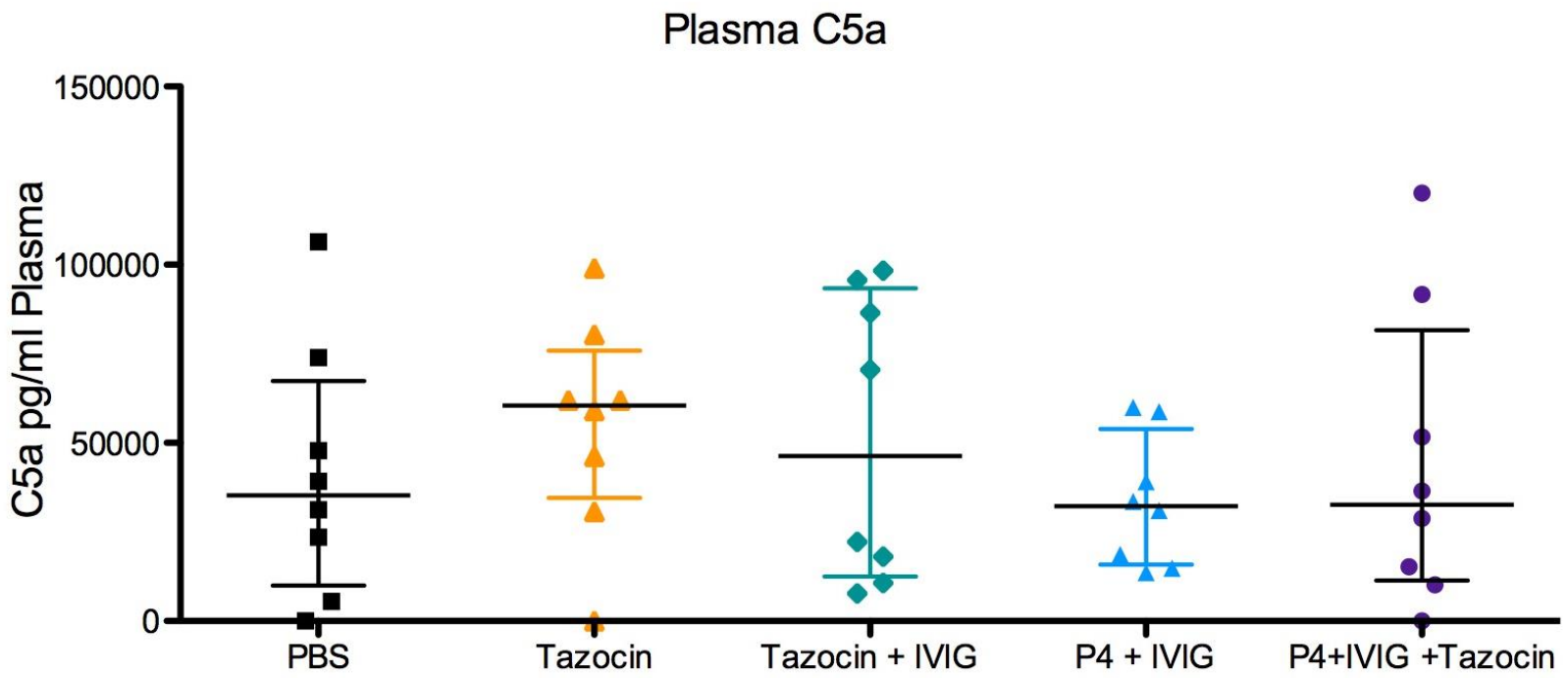


Figure 37 Double dose P4 therapy with antibiotics in *E. coli* infection model – Plasma C5a
Mice n=8 per group were infected with 1 x 10^7 CFU *E. coli* via the intraperitoneal route. 1 and 4 hours post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were culled at seven hours post infection and blood collected for determination of cytokine concentrations in plasma. Plotted as mean and SEM. Analysed by Kruskal-Wallis test and Dunn's multiple comparison test p=0.7613 (*** p<0.0001, ** p<0.005, * p<0.05)

c) Cell populations

Previous work looking at P4 treatment of *S. pneumoniae* infection and administration of P4 to naïve mice has shown that P4 treatment resulted in an increase on the percentage of neutrophils found in the blood as well as an increase in expression of FcγRs on neutrophils; with this in mind I performed experiments to look at the neutrophil populations in the blood of *E. coli* infected mice treated with Tazocin or Tazocin and P4-IVIG in combination (167). In addition to FcγR expression I looked at the expression of CD88 (C5aR) as binding of C5a to this receptor was been linked to neutrophil dysfunction as well as modulation of FcγR expression (115, 128). As with previous experiments mice were infected with 10^7 CFU/mouse of *E. coli* and treated one and four hours post infection before culling at seven hours post infection for blood collection. Blood samples were washed and stained with antibodies before sample acquisition on a BD LSR II flow cytometer.

Significantly higher percentages of neutrophils were seen in Tazocin ($p<0.05$, median = 60%) and P4-IVIG + Tazocin ($p<0.0001$, median = 72%) treated groups when compared to PBS treated mice (median = 31%) (Figure 38). When analysed in isolation the difference between Tazocin and P4-IVIG + Tazocin was statistically significant ($p<0.03$, unpaired t-test). Both high and low levels of circulating neutrophils have been associated with poor prognosis; low neutrophil count indicates possible immunosuppression whereas an overly high neutrophil count is suggestive of excessive inflammation (176).

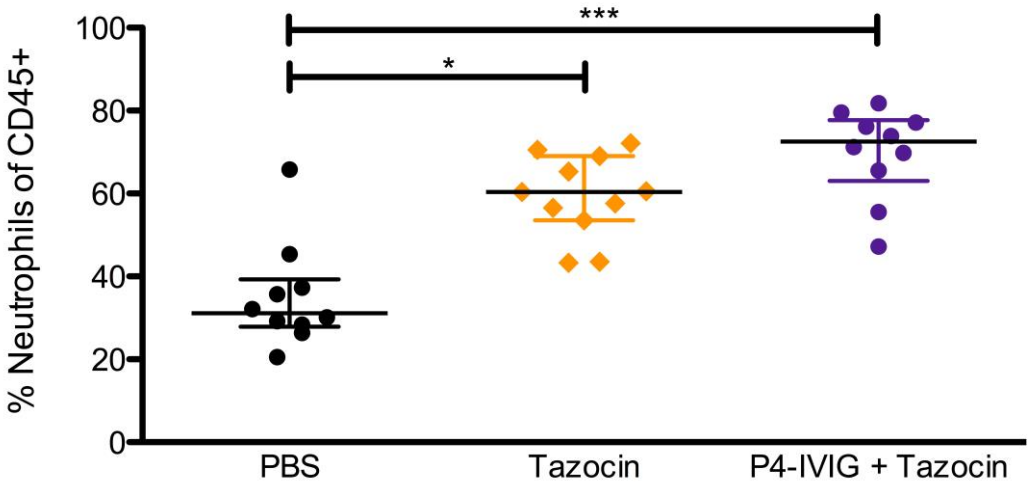


Figure 38 Double dose P4 therapy with antibiotics in *E. coli* infection – % Neutrophil (CD45+, CD11b+, Gr-1+) of CD45+ cells

Mice n=10 per group were infected with 1 x 10^7 CFU *E. coli* via intraperitoneal route. 1 and 4 hours post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were culled at seven hours post infection and blood collected for staining of neutrophils to determine neutrophil (CD45+, CD11b+, Gr-1+) % of CD45+ cells. Plotted as median and IQR. Analysed by Kruskal-Wallis test and Dunn's multiple comparison test *** $p<0.0001$, * $p<0.05$

In order to look at the expression levels of FcγRs on neutrophils, cells were gated using anti-CD45, GR-1 and CD11b antibodies. Expression of CD64 and CD32/16 were measured on these neutrophil populations. CD32/16 antibodies were used, as there is no antibody commercially available that can distinguish between the CD32 and CD16 receptors in mice. No difference was seen in the level of CD64 (FcγRI), between groups although there was a trend towards lower expression with PBS<Tazocin<P4-IVIG + Tazocin; the median value for CD64 expression was highest in PBS treated mice (MFI = 354) followed by Tazocin (MFI = 275) and P4-IVIG + Tazocin had the lowest expression (MFI = 225) (Figure 39- top panel). This decrease in CD64 expression as opposed to being related directly to augmentation of Fc receptor expression by P4 treatment could reflect the severity of infection as high CD64 has been shown clinically to be an indicator of infection and sepsis (177, 178). Expression of CD64 is also highest in immature cells, which could indicate that the PBS treated groups have a higher proportion of immature neutrophils in the blood stream compared with treated groups.

Expression of CD32/16 (FcγRII/III) on neutrophils was significantly higher in both the Tazocin ($p<0.05$, median MFI = 6950) and P4-IVIG + Tazocin ($p<0.005$, median MFI = 7343) groups when compared to the PBS (median MFI = 3918) treated group (Figure 39 - bottom panel). These increased levels of CD32/16 could be due to augmentation of FcγR by P4 peptide treatment but could also be indicative of a more mature neutrophil population in Tazocin and P4-IVIG + Tazocin groups or alternatively a result of CD16 downregulation during sepsis; CD16 decreases with increased infection severity, CD32 expression has not been shown to alter during infection (179).

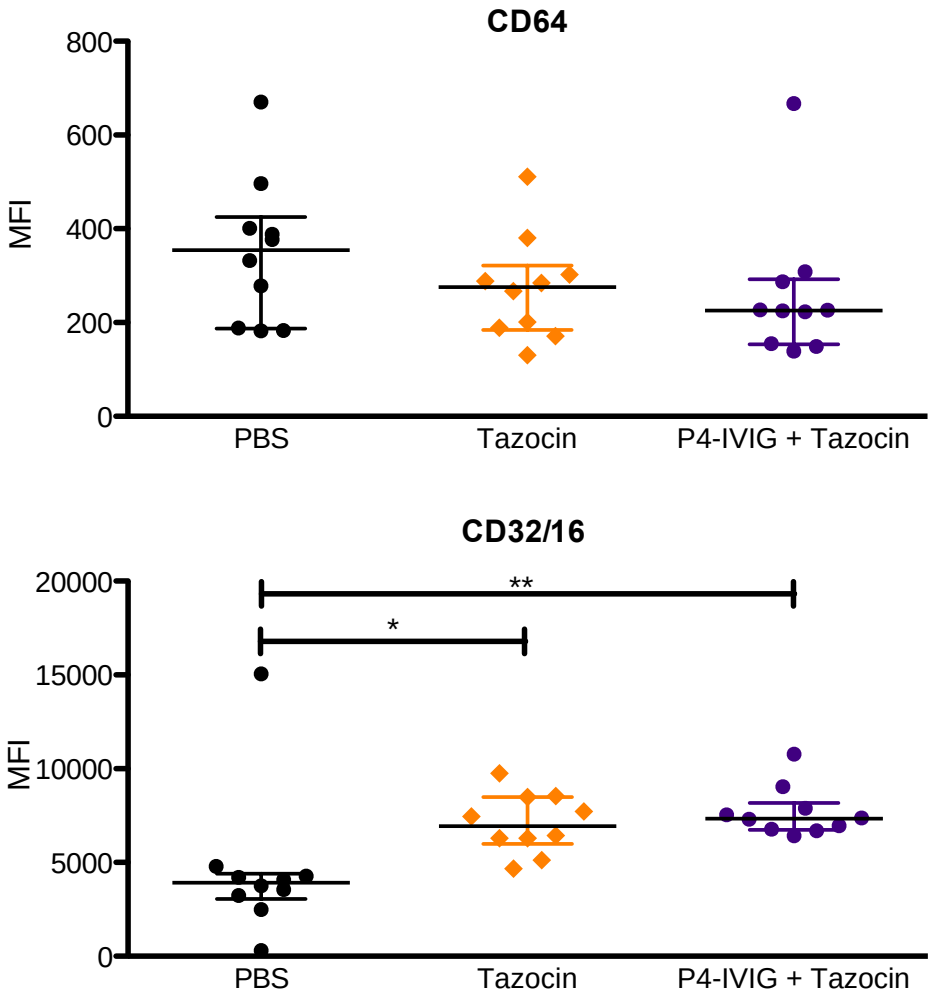


Figure 39 Double dose P4 therapy with antibiotics in *E. coli* infection model – Neutrophil receptor expression FcγRs

Mice n=10 per group were infected with 1 x 10^7 CFU *E. coli* via the intraperitoneal route. One and four hours post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were culled at 7 hours post infection and blood collected for staining of neutrophils (CD45+, CD11b+, Gr-1+) to determine expression levels of CD32/16 (FcγRII/III) and CD64 (FcγRI). Plotted as median and IQR. Analysed by Kruskal-Wallis test and Dunn's multiple comparison test ** $p<0.005$, * $p<0.05$

Expression of CD88 was also measured; isotype controls were used for these experiments as previous studies have reported P4 altering Fc receptor expression, which could effect the measurement of non-Fc recptors through non specific binding of the Fc region to Fcγ receptors (166, 167).

CD88 expression was significantly higher in both the Tazocin (p<0.05, median MFI = 42668) and P4-IVIG + Tazocin (p<0.05, median MFI = 40609) groups when compared to the PBS (median MFI = 24870) treated group (Figure 40). Decreased CD88 is a marker of increased severity and binding of C5a to its receptor can have negative implications for neutrophil function.

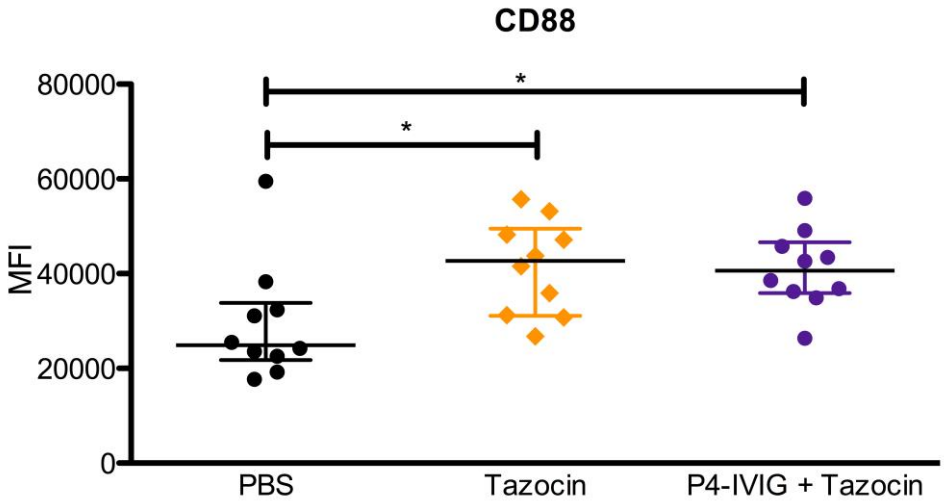


Figure 40 Double dose P4 therapy with antibiotics in *E. coli* infection model – Neutrophil receptor expression CD88

Mice n=10 per group were infected with 1 x 10^7 CFU *E. coli* via the intraperitoneal route. One and four hours post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were culled at 7 hours post infection and blood collected for staining of neutrophils (CD45+, CD11b+, Gr-1+) to determine expression levels of CD88. Plotted as median and IQR. Analysed by Kruskal-Wallis test and Dunn's multiple comparison test * $p<0.05$

d) Endotoxins – LPS

After observing that P4-IVIG and Tazocin treatments only lead to a modest extension in survival time of around 10 hours, despite significant reductions in CFUs in the blood, LPS was measured from the plasma of animals culled at seven hours post infection. LPS is a potent driver of inflammation and plasma levels can be affected by antibiotic treat meant so this was measured to see if it could be a possible factor driving mortality in the model (180).

Plasma endotoxin levels as measured using a *Limulus* amebocyte lysate assay (LAL). As expected endotoxin levels were elevated in all mice (healthy levels should be below detection limit). No significant difference was seen between any of the groups (Figure 41).

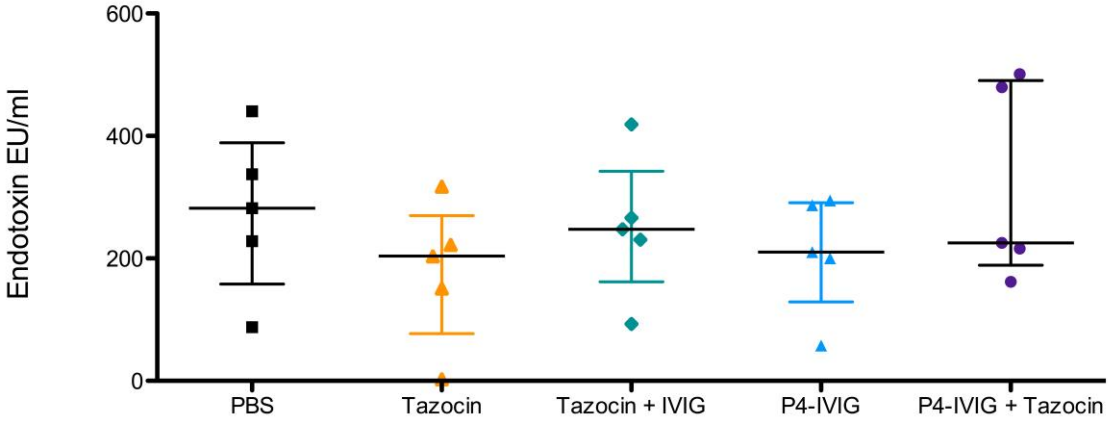


Figure 41 Double dose P4 therapy with antibiotics in *E. coli* infection model – Serum endotoxin levels

Mice n=5 per group were infected with 1 x 10^7 CFU *E. coli* via intraperitoneal route. 1 and 4 hours post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were culled at seven hours post infection and blood collected for determination of endotoxin concentrations in serum. Plotted as median and IQR. Analysed by Kruskal-Wallis test and Dunn's multiple comparison test p=0.4.

E. Results Summary

Treatment with a single dose of P4-IVIG one hour post infection lead to significant reductions in CFUs in both blood and organs at seven hours post infection when compared to PBS treated controls but the number of CFUs in the blood were increasing when compared to the 4 hour time point (Figure 23).

A combination treatment of Tazocin (25% of human equivalent dose) and P4-IVIG at one hour post infection lead to a greater decrease in CFUs compared to either treatment alone with mean CFUs of 7.5×10^7 for Tazocin alone, 2.5×10^7 for P4-IVIG and 8.9×10^5 for the combination treatment, compared to 2.7×10^8 for untreated (PBS) mice (Figure 26). This in turn translated into a significant increase in mean survival over a 24 hour period post infection rising from 8.4 hours for untreated controls to 19 hours for those treated with P4-IVIG + Tazocin, P4-IVIG and Tazocin alone had mean survival times of 16.8 and 15.8 hours respectively. An increase in the percentage of animals surviving to 24 hours was observed, with survival increasing from 0% for untreated controls to 20% for P4-IVIG + Tazocin, survival for P4-IVIG and Tazocin alone survival was 0% (Figure 28).

To determine if host survival could be improved further, an increased dosing schedule was trialled with the addition of a second treatment dose: the first remaining at one hour post infection with the second at four hours post infection.

Increasing the P4 dosing schedule from a single dose to a double dose did not effect the mean survival time over the 24 hours the mice were monitored

but it did effect the survival percentage at 24 hours: P4-IVIG survival increased from 0% to 27%, Tazocin alone from 0% to 11% and P4-IVIG + Tazocin from 20% to 55%, untreated (PBS) survival remained at 0% (Figure 31). The double dose regime also resulted in a greater reduction in mean CFUs in the blood at 7 hours post infection compared with a single dose: Tazocin treatment showing a drop of over 3 logs with a second dose (7.5 x 10^7 to 2.1 x 10^4 CFU/ml), P4-IVIG a modest decrease of just under a log (2.5 x 10^7 to 5.7 x 10^6 CFU/ml) and there was a drop of over 3 logs for P4-IVIG + Tazocin in combination from (8.9 x 10^5 to 816 CFU/ml) (Figure 33).

Treatment with a double dose of P4 peptide in combination with IVIG and Tazocin lead to significantly higher 24 hour survival than treatment with Tazocin alone. However, this combination treatment had equivalent efficacy to Tazocin + IVIG treatment (Figure 31). Between these three treatments there was no significant difference in the CFUs in blood or lungs at seven hours post infection (Figure 33 and Figure 34).

Table 14 Comparison of single and double dose treatment of P4-IVIG in combination with antibiotics- Blood and lung CFUs

Blood 7hr Log CFU					
Treatment	PBS	Tazocin	P4-IVIG	P4-IVIG + Tazocin	Tazocin IVIG
Single Dose	8.19	6.31	7.30	5.35	-
Double Dose	8.50	4.33	6.76	2.91	0.95
Lung 7hr Log CFU					
Treatment	PBS	Tazocin	P4-IVIG	P4-IVIG + Tazocin	Tazocin IVIG
Single Dose	4.93	4.32	4.37	1.85	-
Double Dose	5.44	1.42	5.20	1.10	0.72

Table 14 shows a comparison of CFUs detected in the blood and lungs at seven hours post infection for both single and double doses of treatment. All treatments apart from PBS and P4-IVIG showed a decrease in CFUs. This could suggest that without the antibiotics present a further dose of P4-IVIG does not lead to improvements in bacterial killing, perhaps because the immune response has been exhausted.

Where the three treatments containing Tazocin did differ was the cytokine response to infection; treatment with P4-IVIG +Tazocin resulted in the lowest levels of CXCL1, significantly lover than treatment Tazocin alone (p=0.005). Differences between other pairings of treatment groups were not significant. It should be notes that the level of CXCL1 detected was very high for all treatment groups.

IL-10 levels were significantly higher in PBS and Tazocin treated mice than those treated with Tazocin-IVIG or P4-IVIG. There was no significant difference between IL-10 levels in P4-IVIG + Tazocin treated mice and any other group, with levels sitting midway between the two high groups and the two low groups.

There was no difference in the levels of C5a between treatment groups, with a large degree of spread in the data. Inflammatory marker data is summarised in Table 15.

Table 15 Summary of CXCL1, IL-10 and C5a mean plasma levels at seven hours post infection for mice treated with a double dose of P4-IVIG and antibiotics in combination

Treatment	PBS	Tazocin	P4-IVIG + Tazocin	Tazocin + IVIG	P4 + IVIG
Mean CXCL1 ng/ml	243.8	824.0	181.2	378.8	583.8
IL-10 pg/ml	609.2	499.4	257.8	90.01	57.11
C5a pg/ml	40958	54931	44254	51210	33700

Mice treated with Tazocin or P4-IVIG + Tazocin had a higher percentage of neutrophils in the blood than those treated with PBS (p=0.05 and p=0.0001 respectively) The difference between neutrophil percentages between Tazocin and P4-IVIG + Tazocin treated mice was 12%, if analysised in isolation this difference was significant (p=0.03 t-test).

Expression of receptors on neutrophils from Tazocin and P4-VIG + Tazocin treated groups showed higher levels of both CD32/16 (FcγRII/III) and CD88 than in PBS treated groups. The levels of CD64 (FcγRI) expressed on neutrophils was not significantly different between groups but was lowest in P4-IVIG + Tazocin treated groups followed by Tazocin treated groups, with the highest expression in the PBS treated groups.

To summarise the double dose of P4-IVIG and Tazocin lead to increased survival and decreased bacterial burden compared to a single dose but was not superior to a double dose of Tazocin and IVIG in combination, which also

had high survival over 24 hours (Tazocin-IVIG = 60%, P4-IVIG + Tazocin = 55%) and superior clearance of bacteria.

The difference in the levels of IL-10 and CXCL1 are difficult to interrupt based on a single time point as it is unknown where they are increasing or decreasing as the infection progresses and also how the consumption rate of these molecules is effecting levels detectable in the plasma.

Significant differences in neutrophil percentages and expression of FcγR and CD88 on neutrophil were only seen between groups that received treatment versus PBS controls which could suggest that these difference reflect a difference in infection severity at the given time point as opposed to a drug induced change in receptor expression.

The severity of the model necessitated early sacrifice (in line with the terms of the Home Office licence), thus limiting the opportunity of dosing. To better assess the efficacy of P4-IVIG a less severe model would be of benefit.

F. Discussion

The model of *E. coli* systemic infection developed for this project, was extremely acute, with quite an aggressive progression into sepsis; CFUs were detectable in the blood within one hour of peritoneal infection and PBS treated animals reached lethargy (humane endpoint of infection) within ten hours.

1. Survival and bacterial burden

Single dose treatment with P4 peptide and IVIG lead to a significant reduction in the number of CFUs in the blood, lungs and spleen at seven hours post infection compared to PBS treated mice however the CFUs recovered from these mice was increasing between four and seven hours post infection. With a bacterial burden of around 10^7 CFU/ml in the blood at seven hours post infection these mice were unlikely to survive, for this reason antibiotics were introduced into the model. This model of P4-IVIG treatment in combination with antibiotics is also more in line with how future clinical use is envisaged; with P4 therapy being adjunctive to, not replacing, conventional antimicrobial therapies. Tazocin was chosen after consultation with an ICU physician whom indicated that Tazocin would be the antibiotic of choice for an abdominal infection suspected to be due to a Gram-negative pathogen. The dose of 9 mg/mouse (25% of the human equivalent dose) was selected after initial dose testing confirmed that it was insufficient to clear the infection alone therefore still allowing for an effect of P4 therapy to be shown.

Addition of Tazocin to the model had a synergistic effect with P4-IVIG treatment in combination with Tazocin leading to a decline in CFUs greater

than either of the two separately, with significantly fewer CFUs recovered with P4-IVIG + Tazocin than from treatment with Tazocin alone.

This combination treatment also leads to improvements in survival, all mice treated with Tazocin alone reached lethargy (humane endpoint) with 16 hours post infection, all mice treated with P4-IVIG with 24 hours, whilst 40% of mice treated with the combination of P4-IVIG + Tazocin survived to 24 hours.

At the end of these experiments it was observed that the number of CFUs recovered from Tazocin and P4-IVIG + Tazocin treated mice at the time of death where not as high as would be expected considering the degree of lethargy and were considerably lower than those treated with PBS or P4-IVIG. Alongside this, the rapid onset of symptoms during infection led to the suspicion that endotoxins (LPS) might be playing a significant role in the pathophysiology of the infection.

A second dose was added to the treatment schedule in an attempt to improve outcomes in the model, along with the Tazocin + IVIG, this group was added due to the suspicion that LPS was contributing significantly to the pathophysiology during infection.

Treatment with all three drug combinations that included Tazocin: Tazocin, Tazocin + IVIG and P4-IVIG + Tazocin led to significantly lower numbers of CFUs in the blood, lungs and spleen when compared to PBS treat mice. There wasn't however a statistically significant difference in the number of CFUs recovered the three groups. There was a difference in 24 hour survival time between Tazocin and P4-IVIG + Tazocin in combination. However, this

difference, albeit non-significant, was also evident when treating with Tazocin and IVIG in combination. There was no difference in survival time between P4-IVIG + Tazocin and Tazocin + IVIG.

The reason for a poor survival despite significant reductions in CFUs with Tazocin, P4-IVIG + Tazocin and Tazocin + IVIG treatment was likely due to animal suffering from septic shock. In septic shock patients suffer from intractable vasodilation, microvascular thrombosis, increased adhesion of leukocytes to venules and increased vascular permeability as a result of excessive inflammation, coagulation dysfunction and endothelial damage; all of which lead to impaired tissue perfusion and organ failure (138). These patients would receive interventions to support their failing physiology such as intravenous fluids, vasopressors, inotropic drugs and mechanical ventilation; these measures would be difficult to implement in animal models (181).

2. Inflammatory Markers

a) CXCL1

All mice had very high levels of CXCL1, which has been shown to be essential to survival in polymicrobial sepsis models (cecal ligation and puncture) (169). The levels of CXCL1 were slightly lower in P4-IVIG + Tazocin treated mice than in the other groups, although this difference was only significant between P4-IVIG + Tazocin and Tazocin alone. The difference in median values was ten fold between the two groups. This difference could be interpreted in a number of ways; in experiments with human epithelial cells in tissue culture P4 was shown to decrease the expression of IL-8 (human homolog of CXCL1), so one could conclude that the lower levels of CXCL1 are due to direct modulation of cytokine production, this unlikely to be the case though as there was no reduction seen in mice treated with just P4-IVIG (182). An alternative hypothesis is that there is less CXCL1 because there is a lower bacterial burden in this group, again this is unlikely as the Tazocin + IVIG group, which showed the lowest bacterial burden still had very high levels of CXCL1. Another possibility is that IL-10-mediated attenuation of CXCL1 production, this has been observed in with LPS induced CXCL1 and *Candida albicans* infection, where IL-10 destabilises CXCL1 mRNA and *de novo* synthesis of the protein (183-185). This could be possible as the P4-IVIG + Tazocin treatment group had relatively high of IL-10 and a similar bacterial burden to the Tazocin + IVIG group which had lower IL-10 and higher CXCL1. However the Tazocin only treated group had the second highest level of IL-10 and the highest level of CXCL1. The final theory to explain the lower levels of CXCL1 in the P4-IVIG

+ Tazocin is that it is bound to the CXCR2 receptor on neutrophils and that the higher proportion of neutrophils in the blood of P4-IVIG + Tazocin has resulted in a higher turnover of the cytokine. This could also help explain the disparity between the high neutrophil levels and low CXCL1 in this group. All of these theories may contribute to the lower CXCL1 levels in P4-IVIG + Tazocin but to help delineate which factors are important more time points would be needed in order to establish the temporal order of cytokine production and their effects.

b) IL-10

The highest levels of IL-10 were seen in PBS treated and Tazocin treated mice. High levels of IL-10 in the PBS treated mice seems logical as with high CFUs you would expect high LPS. In the Tazocin treated mice there was also high IL-10 despite these mice having significantly lower numbers of CFUs, this could due to treatment with Tazocin leading to endotoxcin release (112, 186). The P4-IVIG and Tazocin-IVIG group had significantly lower levels of IL-10 than the PBS and Tazocin treated groups, despite the P4-IVIG group higher numbers bacterial and the Tazocin treated group having the possibility of antibiotic-induced endotoxcin release. One possible theory that could explain the lower levels of IL-10 is that the IVIG is mopping up the LPS, the problem with this being the IVIG has been shown to increase IL-10 production by macrophages in response to LPS (187). The intermediate levels of IL-10 in P4-IVIG + Tazocin treated mice are also difficult to explain, neutrophils can be induced to produce IL-10 after direct contact with LPS-stimulated regulatory T-cells and this group did have the highest proportions of neutrophils (188).

With the protective role IL-10 can play in sepsis in mind it would be important to establish the timing of IL-10 induction and cell types producing IL1-0 in this model as well as levels of IL-10 before and after treatment to determine how the various treatments are effecting production (173, 174).

c) C5a

C5a has a very short *in vivo* half-life (2-3 mins) which is thought to be due to the rapid binding and internalisation through C5a receptors so plasma levels of C5a may not reflect the levels being produced *(189)* (190). There was little difference in plasma C5a between group however the PBS group had significantly lower levels of CD88 , which could suggest that it was being produced at higher quantities but had a higher turnover rate.

3. Cell populations

a) Neutrophil percentages

Neutrophil percentages were significantly higher in Tazocin and P4-IVIG + Tazocin treated mice compared to those treated with PBS, with the highest levels being in the P4-IVIG + Tazocin treated group. The factors driving this increase in neutrophils is not clear from this study as the levels of CXCL1 were lowest in the this group and there were no significant differences in the levels of C5a. In further studies it would be important to investigate a broader range of neutrophil chemoattractants as well as cytokines involved in stimulating production of neutrophils such as GM-CSF and G-CSF to see how P4 treatment affects these.

b) FcγR expression

Expression of receptors on neutrophils from Tazocin and P4-VIG + Tazocin treated groups showed a pattern of receptor expression that is indicative of a lower severity of infection compared with the PBS treated group: higher levels of CD32/16 (FcγRII/III), which suggests a more mature neutrophil population and lower levels of CD64 (FcγRI) which suggests the infection is less severe than in PBS treated mice at this time point (178). Decreased CD64 expression could also be interpreted as indirect evidence of increased phagocytosis as the receptor is internalised during phagocytosis (191). CD64 expression also deceases during maturation in the bone marrow, so high levels could again suggest that the PBS treated neutrophils are less mature and those treated with Tazocin and P4-IVIG + Tazocin. Whether these changes can be attributed directly to augmentation of receptor expression by the P4 peptide or are the result of a lower infectious burden is less clear.

c) CD88

Mice treated with Tazocin or P4-IVIG and Tazocin had higher levels of CD88 on their neutrophil. Reduced expression of CD88 during infection has been shown to correlate with increased infection severity and can be used as prognostic markers for survival in sepsis patients (130). Higher levels of CD88 in the Tazocin and P4-IVIG + Tazocin treated groups could therefore be suggestive of a lower infection severity in these groups.

4. Endotoxcin (LPS)

Endotoxin levels in serum were tested to see if different treatments led to different levels of endotoxin being released into the circulation leading to altered levels of inflammation. No difference in endotoxin levels was observed between any of the treatment groups despite significantly different bacterial loads. Although the LAL assay is very sensitive, serum proteins can interfere with the assay rendering it less reliable, hence more testing would be required to determine whether there is a true difference between endotoxin levels (192).

5. Summary

To summarise P4 treatment in this model of *E. coli* infection was successful to an extent; treating with P4-IVIG lead to a decrease in CFUs detected in mice but significant increases in survival time were only seen with the addition of antibiotics and were not superior to treatment with Tazocin and IVIG in combination. Considering the swift decline of health in animals after infection it may be the case that no intervention could rescue these animals.

Chapter III. *In vivo* murine study of effects of P4 peptide during pulmonary *Klebsiella pneumoniae* infection

A. Introduction

The previous chapter focused on an animal model of *E. coli* infection via the peritoneal route; this infection model gave an acute, very severe infection, this chapter goes on to look at P4 therapy in the treatment of *K. pneumoniae* pulmonary infection. *K. pneumoniae* was chosen as the pathogen as it is the second most common cause of Gram negative bloodstream infection after *E. coli,* accounts for 10% of hospital acquired bacterial pneumonias and in regions where it is particularly prevalent (Taiwan and South Africa) it has a higher mortality than *S. pneumoniae* (38-40).

1. Animal Model

For this study an animal model of *K. pneumoniae* respiratory infection (with sepsis secondary to pneumonia) was developed in order to assess treatment efficacy. This model was similar to the *S. pneumoniae* model of invasive pneumococcal disease published by Bangert *et al.* with mortality for untreated subjects at 80-100% and a progression to sepsis between 24 and 30 hours post infection. This model was used for both survival and time pointed experiments. Models utilised also assessed the efficacy of P4 treatment as an adjunctive therapy in combination with antibiotic treatment.

2. Cell surface marker and cytokines

Tissue and blood from time pointed experiments were collected for flow cytometry analysis of cell surface markers and measurement of cytokines in order to monitor the effect of the effect of treatment on the immune response

to infection. The same cell surface and inflammatory markers were used as in Chapter II.

B. Methods and Materials

1. Media preparation

a) Blood Agar

400 ml of distilled water was mixed with 16 g of blood agar base 2 (Oxoid CM0271) before autoclaving at 121°C for 15 minutes. Once the agar had cooled to approximately 56°C, 20 ml of defibrinated horse blood (Oxoid SR0050) was added to give a final concentration of 5% blood agar and then poured into sterile petri dishes. Plates were stored at 4°C until use.

b) Brain Heart Infusion Broth

400 ml of distilled water was mixed with 14 g of brain heart infusion (BHI) broth powder (Oxoid CM1135) before autoclaving at 121°C for 15 minutes. BHI broth was cooled before use and stored at room temperature.

For BHI with serum – an aliquot of heat inactivated foetal bovine serum (FBS – Sigma F9665) was thawed and added to broth aseptically at the appropriate concentration.

2. Bacterial strain and inoculum preparation

K. pneumoniae ATCC 43816, a serotype 2 strain was purchased from LGC Standards, UK.

Infections were carried out with mid-log cultures. Strains were streaked on to 5 % blood agar plates from frozen stocks and incubated at 37°C for 16-18 hours. The following day universal tubes containing 5 ml of BHI broth were

inoculated from the plate and incubated at 37°C, 200 rpm for 16-18 hours. Following the 16-18 hour incubation 500 µl was subcultured into 20 ml of LB broth and adjusted to an OD_{600} of 0.1. The culture was incubated at for 37°C, 200 rpm for 1-2 hours until reaching an OD_{600} of 0.5, cultures were centrifuged at 3000 rpm for 5 minutes, the supernatant discarded and the pellet resuspended in PBS, this was repeated and the culture then resuspended at 2 x 10^6 CFU per ml. Doses were plated onto 5 % blood agar to confirm inoculum CFUs .

3. Mouse Strains

Female CD-1 mice aged between 6-7 weeks were used for all experiments.

4. Infection and monitoring

The prepared inoculum was administered by intranasal instillation of 50 µl dose distributed evenly between the two nares (detailed in section II.B). Mice were monitored at regular intervals from administration of infectious dose. Animals were culled when they reached ++ lethargy (details of scoring in methods in section II.B).

5. P4 treatment

P4 peptide and IVIG were administered at 24 and 30 hours post infection via a 50 µl intravenous injection into the tail vein at a concentration of 2 mg/ml dissolved in DEPC treated water. IVIG was administered by a 100 µl intraperitoneal injection of Gamunex-C (Grifols, Spain), 100 mg of protein per ml. Control animals received an injection of equivalent volume of sterile PBS.

6. Antibiotic treatment

Tazocin (Pfizer, USA) – piperacillin/tazobactam – was used in experiments at a dose of 9 mg/mouse, which equated to ¼ of the human equivalent dose. Tazocin was injected via the tail vein, 50 µl dissolved in DEPC treated water at the same time points as P4 treatment. Control animals received an injection of equivalent volume of sterile PBS.

Animal procedures, tissue collection, CFUs and flow cytometry were performed as detailed in section II.B

C. Results

1. Survival

The efficacy of P4 treatment in combination with the antibiotic (Tazocin) was investigated during severe pneumonia (with secondary sepsis) caused by *K. pneumoniae*. Disease progression during this infection model is similar to that of *Streptococcus pneumoniae* so I used the same dosing schedule described by Bangert *et al*. (167). Doses of P4, IVIG and Tazocin were administered at 24 and 30 hours post infection, with P4 and Tazocin both being given intravenously and IVIG via intraperitoneal injection. Survival was monitored over a seven day period with surviving mice being culled at this point to confirm clearance of infection.

The P4-IVIG + Tazocin group showed 70% survival at day seven compared with 20% for PBS and Tazocin groups and 30% for P4-IVIG and Tazocin-IVIG groups (Figure 42).

The mean survival time (p=0.04) Mean survival times for P4-IVIG + Tazocin were significantly longer compared to PBS treated mice (PBS mean survival = 78.3 hours, P4-IVIG + Tazocin mean survival time = 148.2 hours, p<0.05). Survival times for Tazocin (98.2 horus), Tazocin + IVIG (95.4 hours) and P4-IVIG (93.8 hours) were not significantly higher than PBS treated mice (Figure 43).

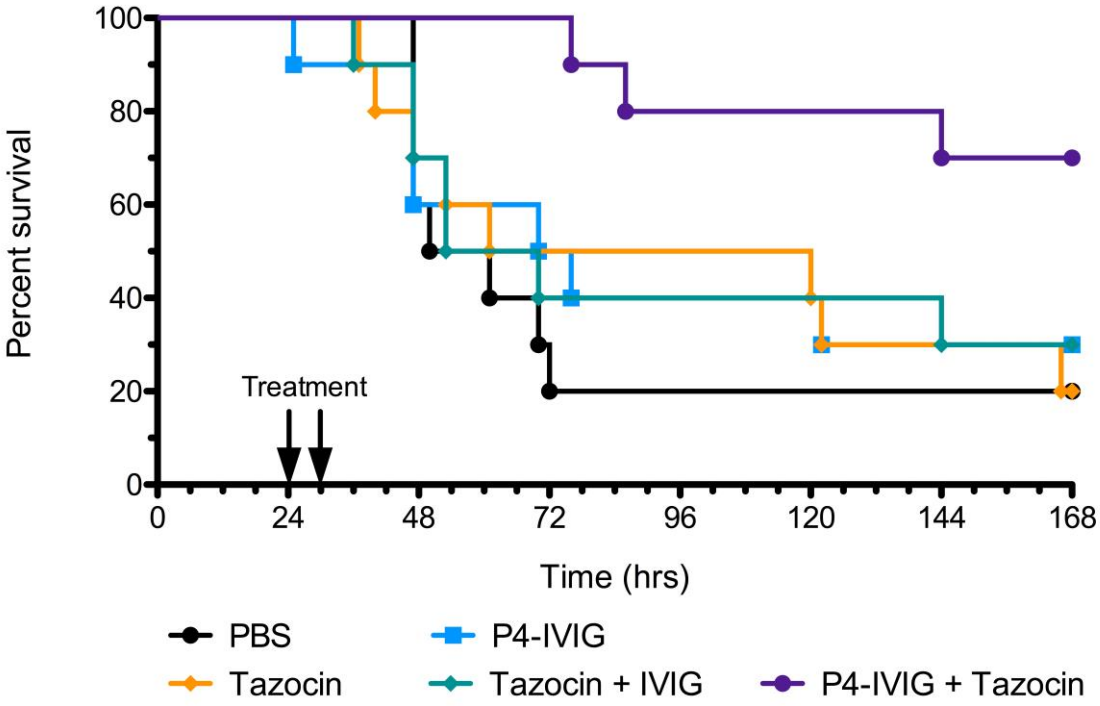


Figure 42 Double dose P4 therapy with antibiotics in *K. pneumoniae* infection model - Survival

Mice n=10 per group were infected with 1 x 10^5 CFU *K. pneumoniae* via intranasal instillation. 24 and 30 hours post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were monitored for signs of disease and culled when they reached ++ lethargic.

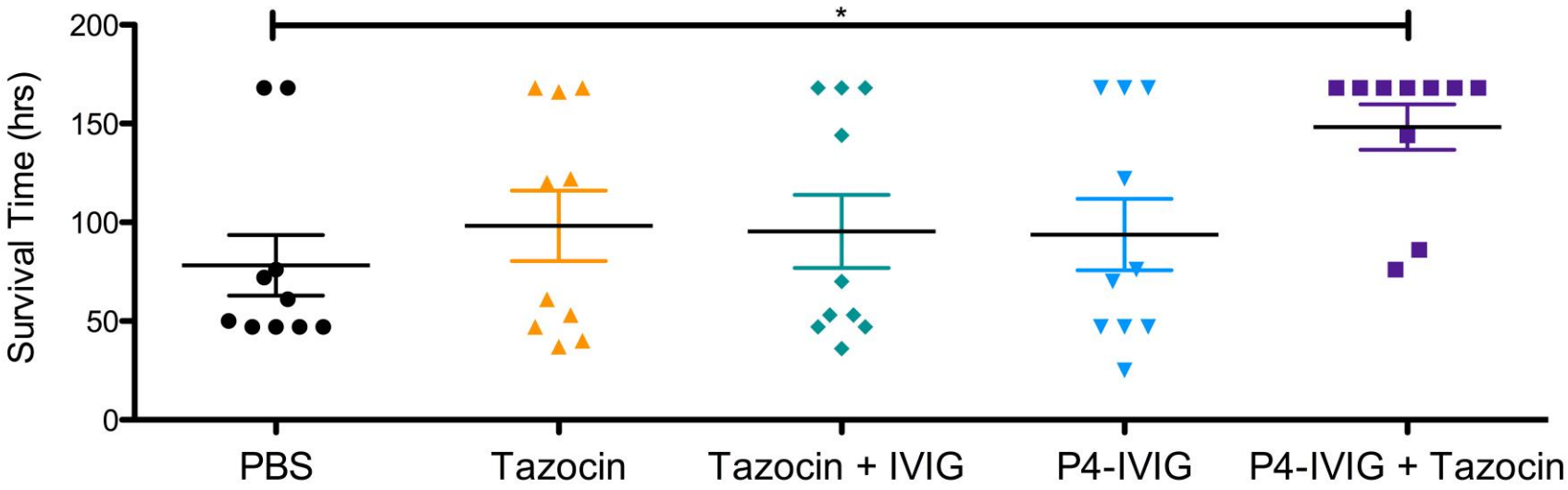


Figure 43. Double dose P4 therapy with antibiotics in *K. pneumoniae* infection model – Survival
Mice n=10 per group were infected with 1 x 10^5 CFU *K. pneumoniae* via intranasal instillation. 24 and 30 hours post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were monitored for signs of disease and culled when they reached ++ lethargic. Survival time in hours with mean and SEM. Analysed by one-way ANOVA test, p=0.0482 and Bonferroni's post test * = p<0.05.

2. Bacterial Burden

36 hours was chosen as the time point to assess bacterial burden as it is the last point at which there is 100% survival in all treatment groups.

Both P4-IVIG + Tazocin treated mice (median CFUs = 0.0, IQR = 0 – 1.1 x 10^4) and those treated with Tazocin alone (median CFUs = 2.4 x 10^3, IQR = 3 x 10^2 – 8 x 10^3) showed significant reductions in blood CFUs compared with PBS treated mice (median CFUs = 1.6 x 10^5, IQR = 2.0 x 10^4 – 3.6 x 10^6) (Figure 44- top).

Both P4-IVIG + Tazocin treated mice (median CFUs = 3.333 x 10^5, IQR = 1.3 x 10^5 – 7.5 x 10^5) and those treated with Tazocin alone (median CFUs = 4.0 x 10^5, IQR = 3.8 x 10^4 – 3.8 x 10^6) also showed significant reductions in lung CFUs compared with PBS treated mice (median CFUs = 1.3 x 10^7, IQR = 7.3 x 10^6 – 1.6 x 10^8) (Figure 44 - bottom).

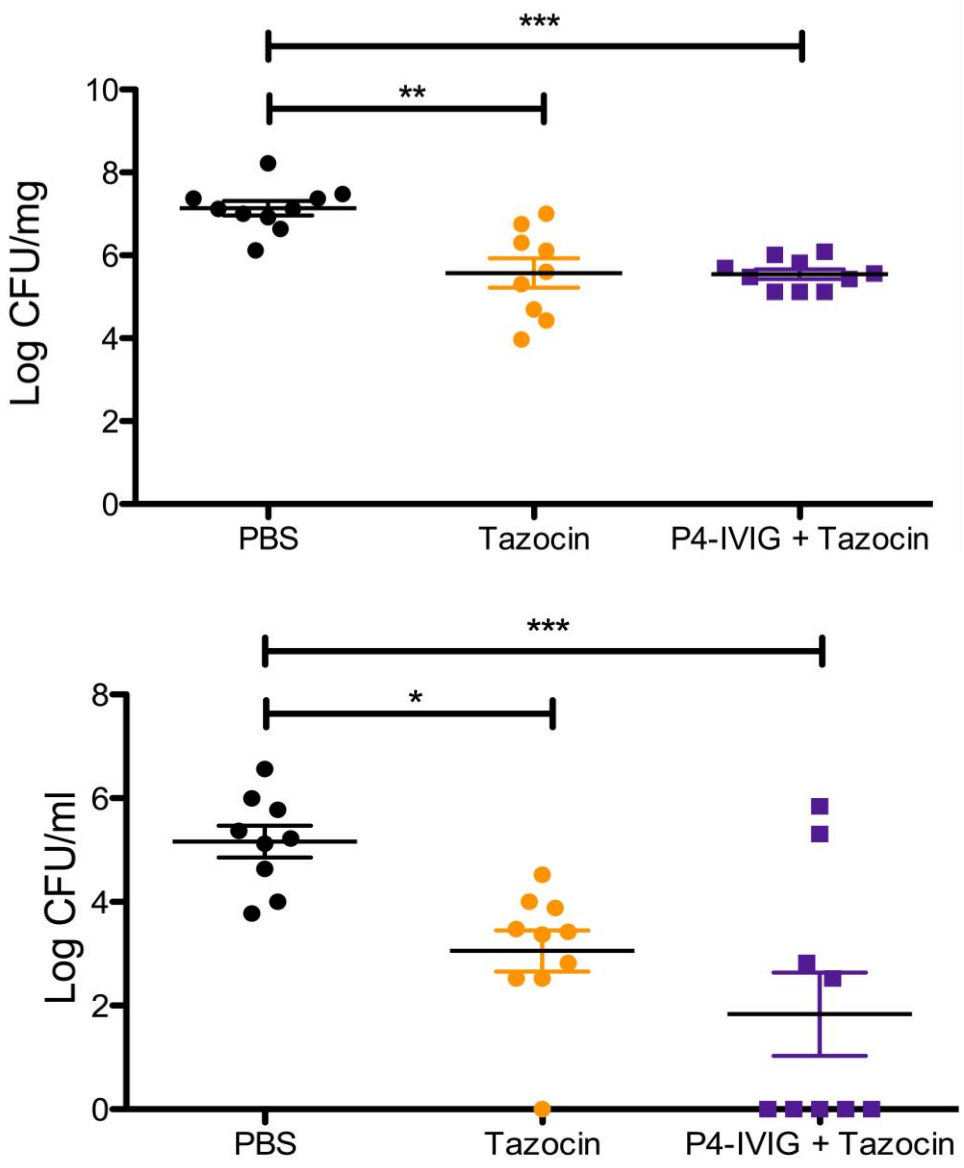


Figure 44. Double dose P4 therapy with antibiotics in K. pneumoniae infection model – Blood and Lung CFUs

Mice n=10 per group were infected with 1 x 105 CFU *K. pneumoniae* via intranasal instillation. 24 and 30 hours post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume.

Top:Mice were monitored for signs of disease and culled at 36 hours post infection. Blood was collected for determination of CFUs. Analysed by Kruskal-Wallis test and Dunn's multiple comparison test p=0.002, *** p<0.0001, * p<0.05.

Bottom: Lung tissue was collected for determination of CFUs. Analysed by Kruskal-Wallis test and Dunn's multiple comparison test p=0.0002, *** p<0.0001, ** p<0.005.

3. Inflammatory Markers

a) Plasma

No significant differences were observed in CXCL1 (p=0.5), MIP-2 (p=0.2), or C5a (p=0.7) concentrations in plasma between the three groups. Plasma IL-10 concentration was also measured but was below the level of detection of the assay (2 pg/ml) (Figure 45 and Figure 46).

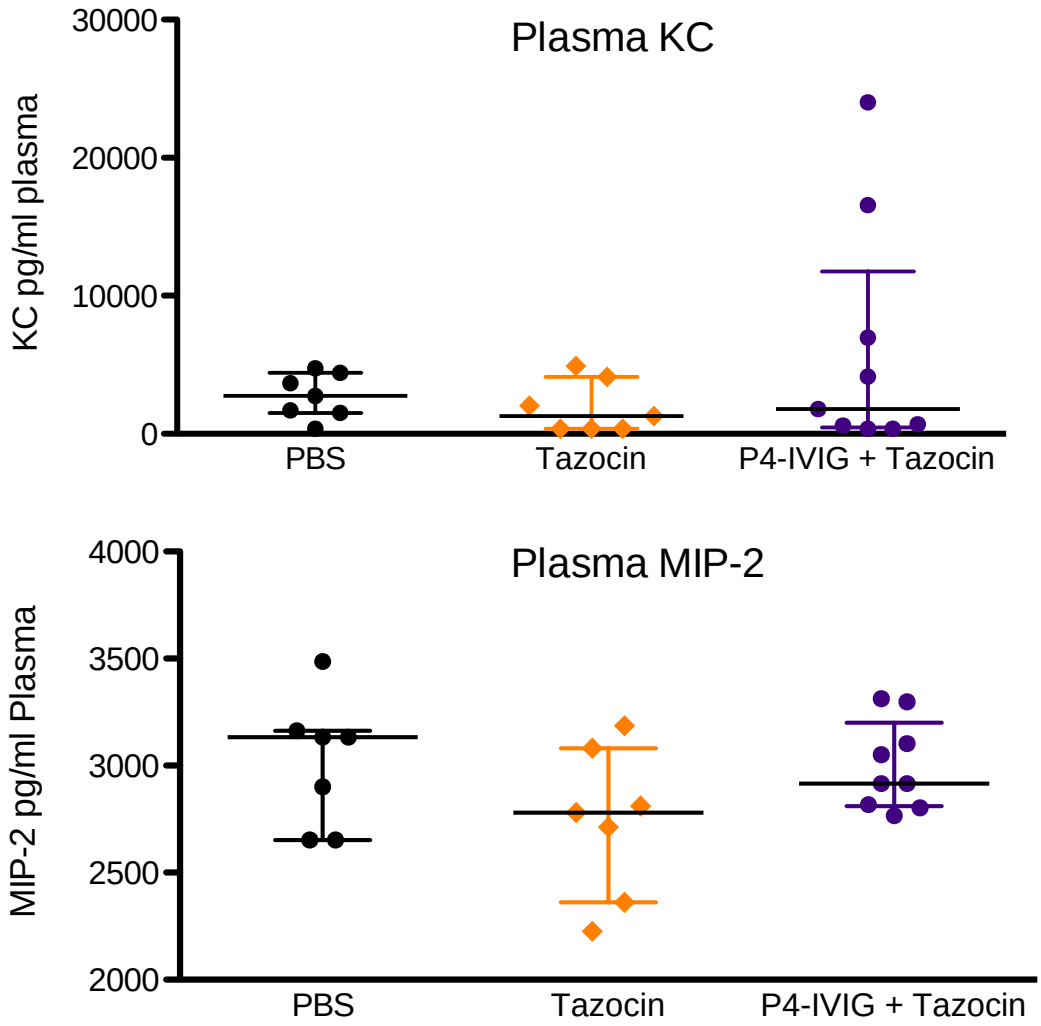


Figure 45. Double dose P4 therapy with antibiotics in *K. pneumoniae* infection model – Inflammatory markers in plasma (CXCL1 and MIP-2)
Mice n=10 per group were infected with 1 x 10^5 CFU *K. pneumoniae* via intranasal instillation. 24 and 30 hours post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were monitored for signs of disease and culled at 36 post infection. Blood was collected for determination of plasma cytokine concentrations. Plotted as median and IQR. Analysed by Kruskal-Wallis test and Dunn's multiple comparison test -CXCL1 p=0.5, MIP-2 p=0.2

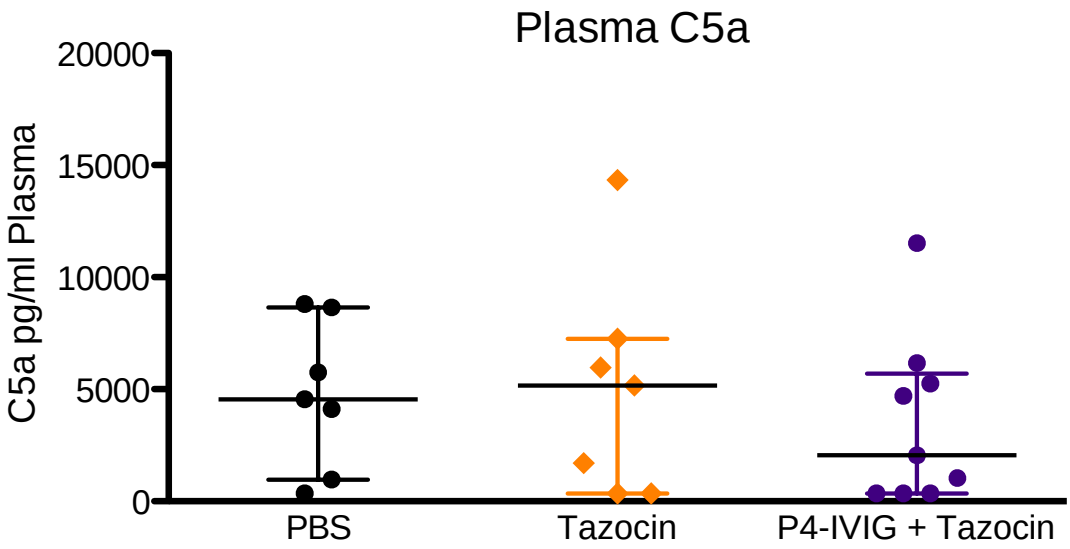


Figure 46 Double dose P4 therapy with antibiotics in *K. pneumoniae* infection model – Inflammatory markers in plasma (C5a)
Mice n=10 per group were infected with 1 x 10^5 CFU *K. pneumoniae* via intranasal instillation. Twenty-four and 30 hours post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were monitored for signs of disease and culled at 36 post infection. Blood was collected for determination of plasma cytokine concentrations. Plotted as median and IQR. Analysed by Kruskal-Wallis test and Dunn's multiple comparison p=0.7

b) Lungs

.No significant differences were seen between CXCL1 concentrations in the lungs (p=0.2) however there was a difference for MIP-2. The levels of MIP-2 in the lungs were significantly higher in PBS treated mice compared to those treated with Tazocin (p=0.005), there was not significant difference between the levels in P4-IVIG + Tazocin and the other two groups (Figure 47).

Tazocin treated mice had significantly higher levels of C5a (mean C5a = 373 pg/ml) than those treated with PBS (mean C5a = 181 pg/ml p<0.05), they also had higher levels than P4-IVIG + Tazocin treated mice (mean C5a = 256 pg/ml) but this difference was not statistically significant (Figure 48).

IL-10 concentrations in the lung, levels were significantly lower (p<0.05) in both P4-IVIG + Tazocin (mean IL-10 = 854 pg/ml) and Tazocin (mean IL-10 = 875 pg/ml) treated mice when compared to PBS (mean IL-10 = 2747 pg/ml) treated mice (Figure 49).

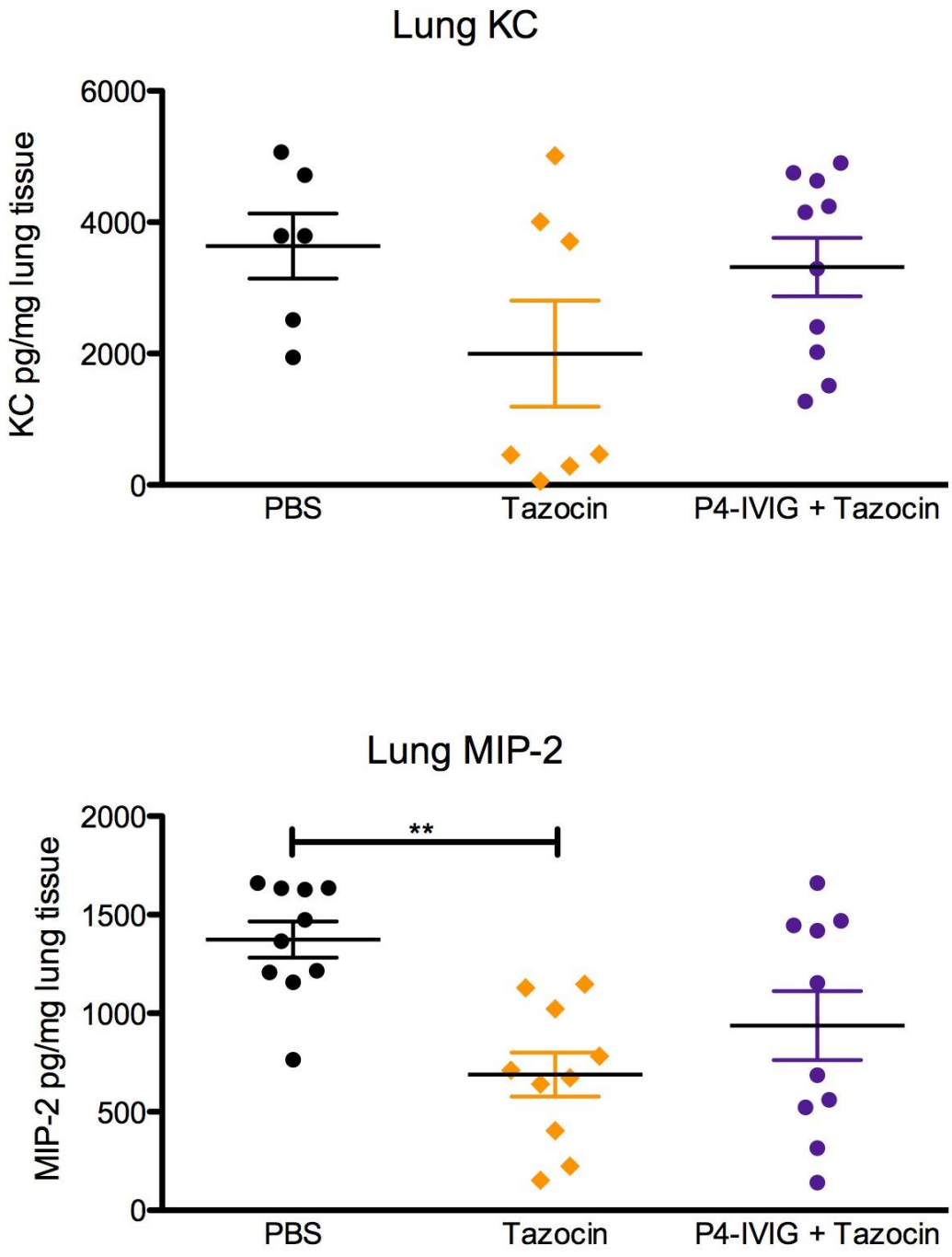


Figure 47 Double dose P4 therapy with antibiotics in *K. pneumoniae* infection model – Inflammatory markers in Lung (CXCL1 and MIP-2)
Mice n=10 per group were infected with 1 x 10^5 CFU *K. pneumoniae* via intranasal instillation. 24 and 30 hours post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were monitored for signs of disease and culled at 36 post infection. Lung tissue was collected for determination of cytokine concentrations. Plotted as mean and SEM. Analysed by one-way ANOVA and Bonferroni post test –CXCL1 p=0.2, MIP-2 p=0.003 ** p<0.005.

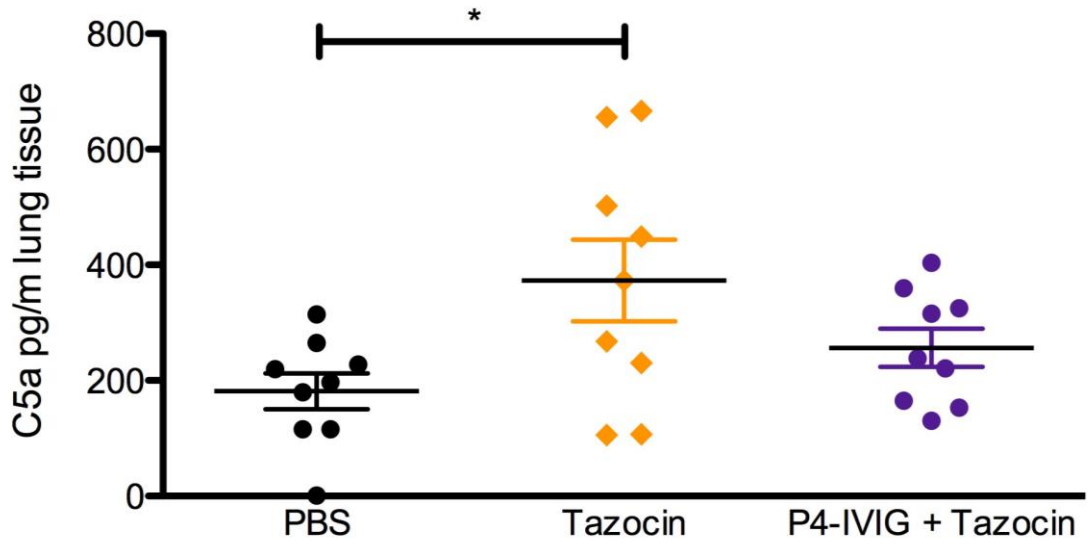


Figure 48 Double dose P4 therapy with antibiotics in *K. pneumoniae* infection model – lung (C5a)

Mice n=10 per group were infected with 1 x 10^5 CFU *K. pneumoniae* via intranasal instillation. 24 and 30 hours post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were monitored for signs of disease and culled at 36 and 48 hours post infection. Lung tissue was collected for determination of cytokine concentrations. Plotted as mean and SEM. Analysed by one-way ANOVA and Bonferroni post test p=0.03, * p<0.05.

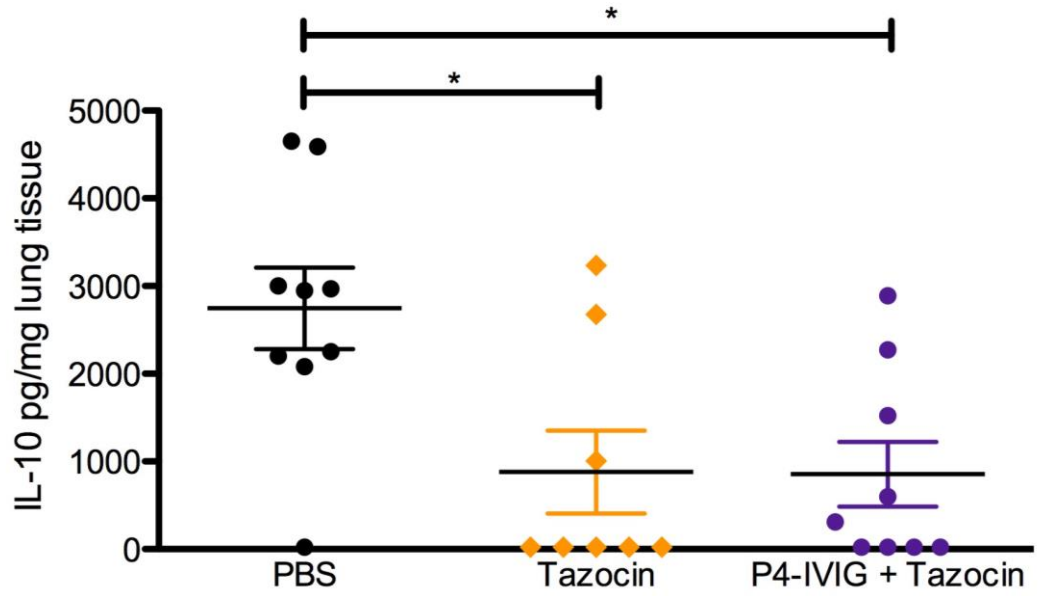


Figure 49 Double dose P4 therapy with antibiotics in *K. pneumoniae* infection model – Lung IL-10

Mice n=10 per group were infected with 1 x 10^5 CFU *K. pneumoniae* via intranasal instillation. 24 and 30 hours post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were monitored for signs of disease and culled at 36 and 48 hours post infection. Lung tissue was collected for determination of cytokine concentrations. Plotted as mean and SEM. Analysed by one-way ANOVA and Bonferroni post test p=0.006 * p<0.05.

4. Cell populations and receptor expression

a) Neutrophils

Neutrophils were defined as CD45+, CD11b+ and Gr-1+ cells, neutrophil percentages were calculated as percentage of all CD45+ cells.

A higher percentages of neutrophils were detected in the blood of P4-IVIG + Tazocin treated mice (mean = 44.13 %) at 36 hours post infection compared to Tazocin (mean - 27.55 %) and PBS (mean = 30.33 %) treated mice (both $p<0.005$) (Figure 50, top panel). Percentages of neutrophils found in the lung tissue at 36 hours post infection showed no significant difference between treatment groups ($p=0.2145$) (Figure 50, bottom panel).

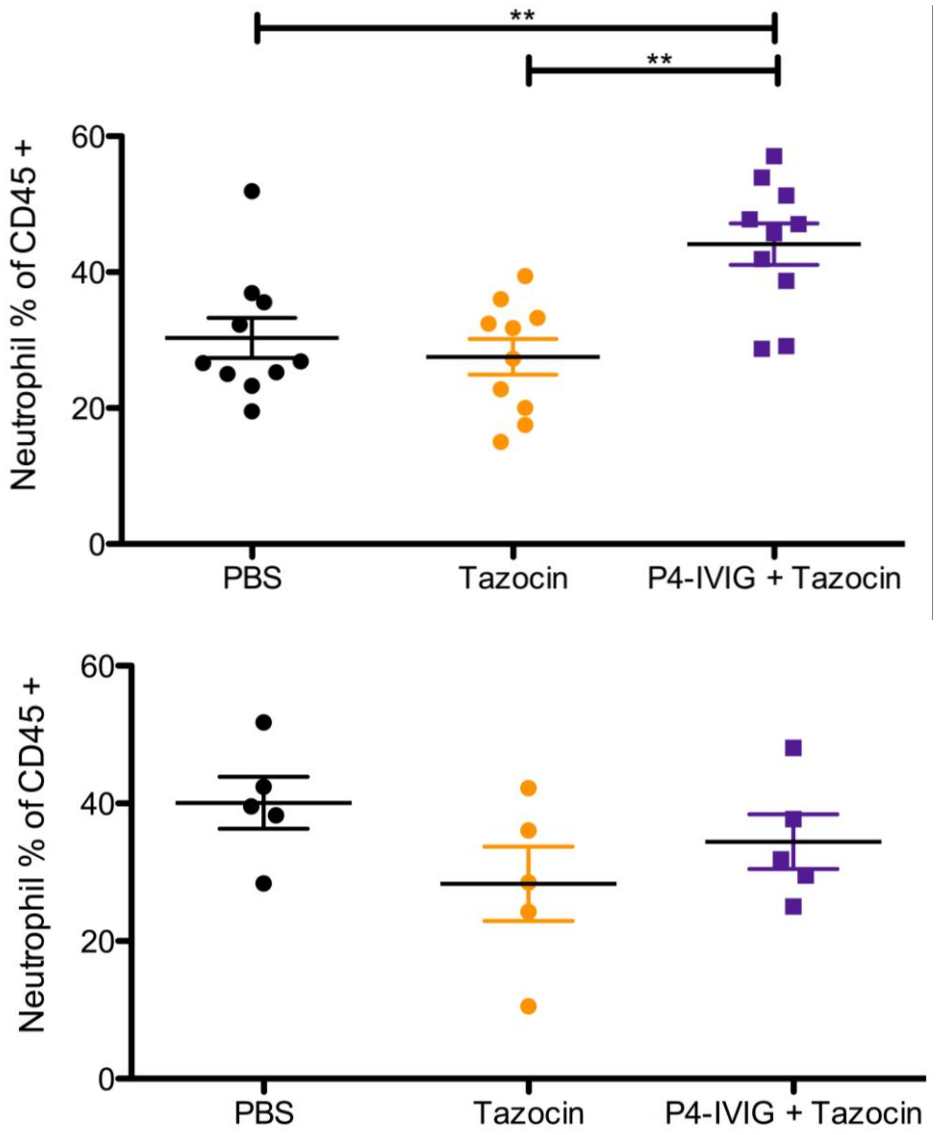


Figure 50 Double dose P4 therapy with antibiotics in *K. pneumoniae* infection model – percentage neutrophils in blood and lungs

Mice n=5 per group were infected with 1 x 10^5 CFU *K. pneumoniae* via intranasal instillation. 24 and 30 hours post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were monitored for signs of disease and culled at 36 post infection.

Top: Blood was collected, red blood cells lysed and cells stained for subsequent analysis by flow cytometry, neutrophils were isolated as CD45+, Gr-1+ and CD11b+ cells. Plotted as mean and SEM. Analysed by one-way ANOVA and Bonferroni post test p=0.0008, ** p<0.005.

Bottom: Lung tissue was collected, processed to a single cell suspension, red blood cells lysed and cells stained for subsequent analysis by flow cytometry, neutrophils were isolated as CD45+, Gr-1+ and CD11b+ cells. Plotted as median and IQR. Analysed by Kruskal-Wallis test p=0.2

b) Receptor expression

The expression of cell surface receptors on neutrophils in the blood at 36 hours post infection was measured. No difference was observed in the expression of CD64 (FcγRI) (p=0.8) or CD88 (p=0.7) although a statistically significant difference (p=0.02) was observed for CD32/16 (FcγRII/III); P4-IVIG + Tazocin treated mice had higher levels of expression than Tazocin or PBS (p=0.05) treated mice (mean MFI for P4-IVIG + Tazocin = 17277, Tazocin = 13337 and PBS = 11581) (Figure 51 and Figure 52).

Receptors CD11b (MAC-1, CR3) and CD35/21 (CR1/2) were also measured but displayed no difference in expression between groups (p= 0.8 and p=0.1 respectively).

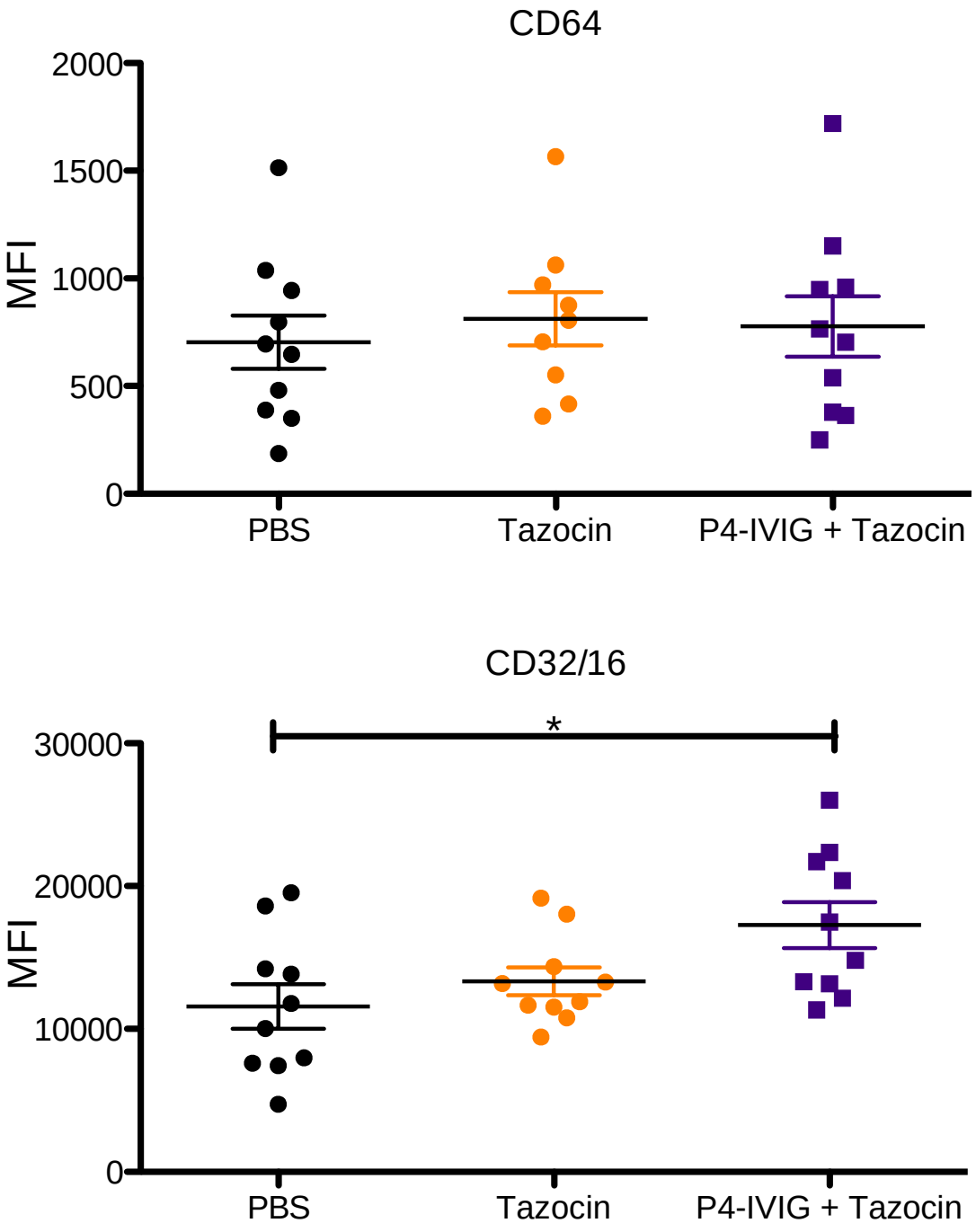


Figure 51 Double dose P4 therapy with antibiotics in *K. pneumoniae* infection model – blood neutrophil receptor expression (FcγR)
Mice n=10 per group were infected with 1 x 10^5 CFU *K. pneumoniae* via intranasal instillation. 24 and 30 hours post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were monitored for signs of disease and culled at 36 hours post infection. Blood was collected, red blood cells lysed and cells stained for subsequent analysis by flow cytometry. Analysed by one-way ANOVA and Bonferroni post test –CD64 (FcγRI) p=0.8, CD32/16 (FcγRII/III) p=0.02, * p<0.05.

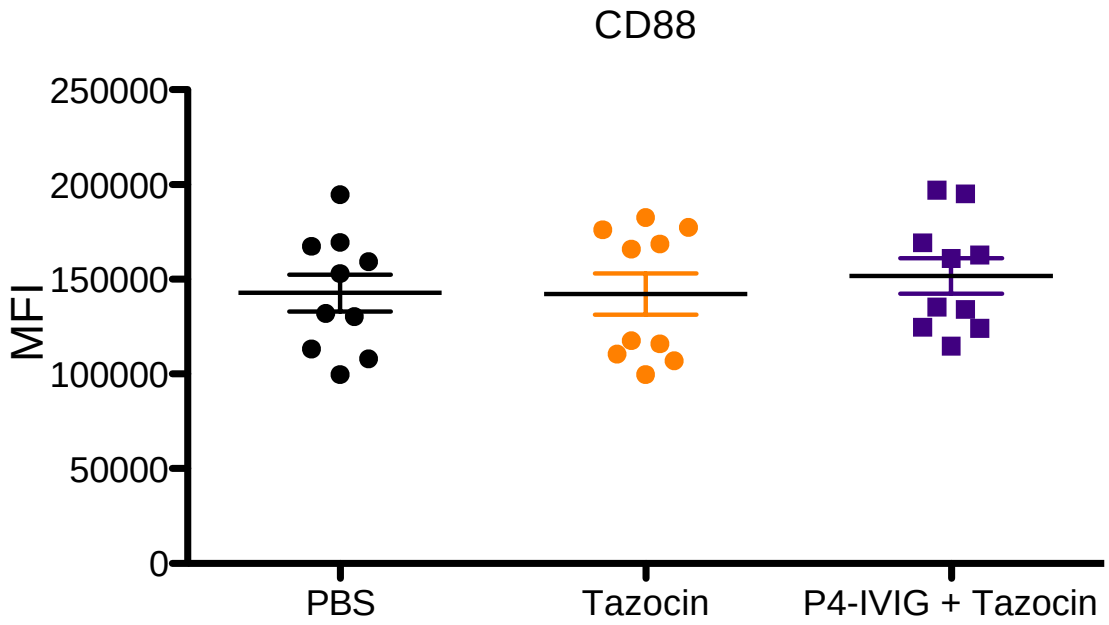


Figure 52 Double dose P4 therapy with antibiotics in *K. pneumoniae* infection model – blood neutrophil receptor expression C5aR

Mice n=10 per group were infected with 1 x 10^5 CFU *K. pneumoniae* via intranasal instillation. 24 and 30 hours post infection mice were treated I.V. with 50 µl of P4 peptide (2 mg/ml) and/or Tazocin (9 mg/mouse), I.P. with 100 µl of IVIG (100 mg/ml). Control mice received PBS injections of equal volume. Mice were monitored for signs of disease and culled at 36 post infection. Blood was collected, red blood cells lysed and cells stained for subsequent analysis by flow cytometry. Analysed by one-way ANOVA and Bonferroni post test p=0.7.

D. Results Summary

Treatment with a double dose of P4-IVIG + Tazocin led to higher percentage survival (70%) over 7 days than treatment with Tazocin alone (20%) and Tazocin+ IVIG (30%). Mean survival time was also significantly longer in P4-IVIG + Tazocin treated mice compared with PBS treated mice ($p<0.05$).

When CFUs were compared for P4-IVIG + Tazocin, Tazocin and PBS, treatment with both P4-IVIG + Tazocin and Tazocin alone led to a significant reduction in CFUs compared to control group. While decreases were similar in the lungs, the P4-IVIG + Tazocin treated group showed a far greater decrease in CFUs in the blood with 50 % of combination treated mice having no detectable CFUs in the blood at 36 hours.

These three treatments showed no difference in plasma cytokine levels the levels however the level of C5a in the lungs were significantly higher for mice treated with Tazocin alone and the levels of IL-10 in the lung were highest for mice treated with PBS.

The percentage of neutrophils found in the blood of P4-IVIG + Tazocin treated mice was significantly higher than for untreated controls and those treated with Tazocin alone.

Expression of receptors on neutrophils from all groups showed similar levels of both CD64 and CD88 but differed in their expression of CD32/16. No differences were observed in receptor expression levels in the lung nor were there any difference in neutrophil percentage observed.

E. Discussion

1. Survival and bacterial burden

Treatment with P4 peptide, IVIG and Tazocin lead to an increase in host survival (70%) when compared to both infected but non-treated control (20%) mice and those that received Tazocin (20%) or Tazocin in combination with IVIG (30%). Along with an increase in survival, mice treated with P4-IVIG + Tazocin in combination, saw a significant reduction in CFUs, this reduction in the lungs was similar to those treated with Tazocin alone but reductions in the blood were considerably higher with 50% of mice having no CFUs detectable at 36 hours post infection. This reduction in CFUs in blood is likely related to the increase in circulating neutrophils along with their higher level of CD32/16 expression.

2. Inflammatory Markers

a) Plasma

No differences were observed in the levels of cytokine in the plasma of mice at 36 hours post infection. Whether differences would have be observed at an earlier time point, closer to the treatment times, is something that should be considered for further studies.

b) Lungs

Differences were observed in the levels of MIP-2, C5a and IL-10 in lung. The levels of CXCL1 in the lungs were not significantly different between treatment groups.

PBS treated mice had significantly higher levels of MIP-2 compared with Tazocin treated mice. This higher level of MIP-2 is likely due to a higher bacterial burden in the lung.

Levels of C5a in the lung were highest for Tazocin treated mice, why this would be is unclear, expression of CD88 on lung neutrophils doesn't suggest higher binding of C5a in the PBS or P4-IVIG treatment groups.

The concentration of IL-10 in the lungs of PBS treated mice was significantly higher than in either Tazocin or P4-IVIG + Tazocin treated mice. *K. pneumoniae* capsule is known to induce IL-10 during pulmonary infection so the higher levels of bacteria in the lung of PBS treated mice could account for the higher levels of IL-10 (193).

3. Cell populations

a) Neutrophils percentages

As seen with the pneumococcal models of invasive disease there was not a significant increase in lung neutrophils with intravenous P4 treatment (167).

There was however a significantly higher percentage of neutrophils in the blood with P4-IVIG + Tazocin treatment compared with treatment with Tazocin or PBS. This higher proportion of neutrophils in the blood probably helped contribute to the reduction in CFUs seen in the blood.

b) FcγR expression

No differences were observed in the FcγR expression in the lung neutrophils. There was however a difference in the CD32/16 (FcγRII/III) expression in the

blood neutrophils, P4-IVIG + Tazocin treated mice had significantly higher levels of CD32/16 than PBS treated mice and higher expression than mice treated with Tazocin although this difference was not statistically significant. Higher levels of CD32/16 suggest more functionally mature cells and the potential for higher rates of phagocytosis, which is in line with the lower bacterial burden in the blood of these mice; over 50% of the mice had no detectable CFUs in the blood at 36 hours post infection.

c) CD88

No difference in CD88 expression was observed in either lung or blood neutrophils. This fits with the low levels of C5a found in the plasma and lung.

4. Summary

In summary treatment with P4-IVIG and Tazocin in combination was a successful treatment in the *K. pneumoniae* infection model where, unlike in the *E. coli* infection model, the combined therapy was superior to Tazocin-IVIG treatment both in terms of survival and bacterial clearance.

Chapter IV. *Ex vivo* study of the effect of P4 peptide on neutrophils of patients with severe community acquired pneumonia

A. Introduction

1. Community Acquired Pneumoniae (CAP)

Lower respiratory tract infections (LRTIs) and pneumonia are a major cause of mortality or morbidity across the globe; especially prevalent in low and middle-income countries, and the leading infectious cause of death in high-income countries (194, 195). As well as high costs in terms of health, LTRIs are also a substantial economic burden on healthcare systems with European inpatient care costs for community acquired pneumonia (CAP) alone estimated at over €5 billion per year (196).

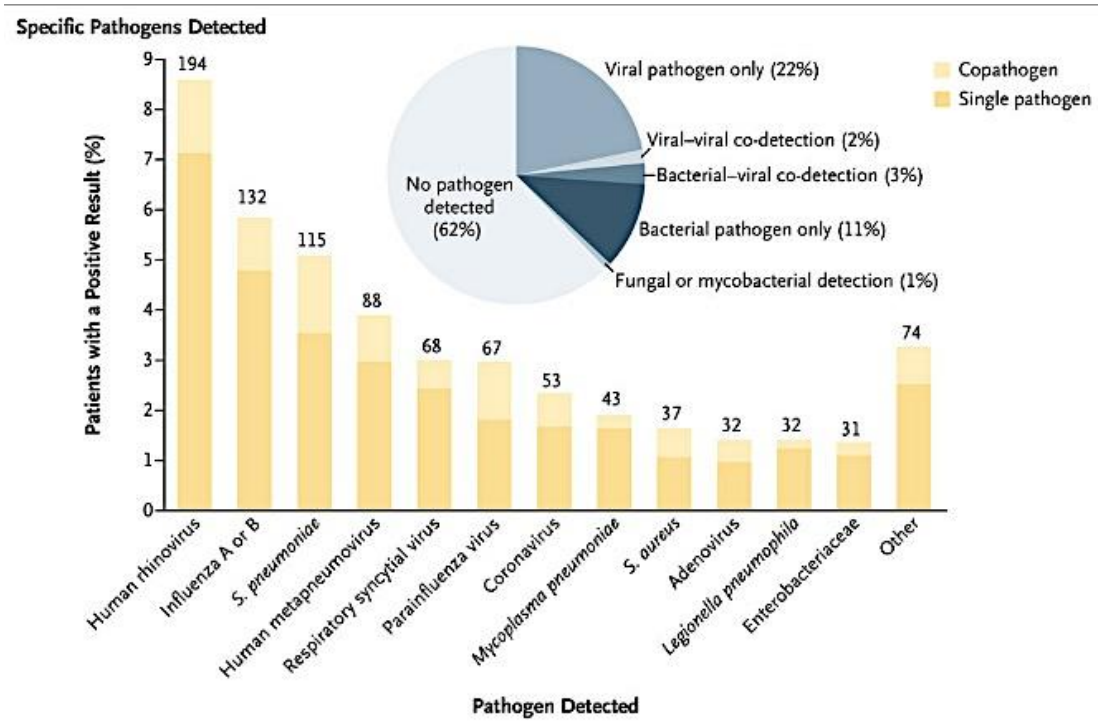


Figure 53 Pathogen Detection among U.S. Adults with Community-Acquired Pneumonia Requiring Hospitalization, 2010–2012.
From Jain et al. 2015 (37).

Streptococcus pneumoniae is the most common cause of bacterial CAP requiring hospitalisation in recent reports from the US (Figure 53)(37).

The etiology of CAP varies greatly between regions and age groups, and is also influenced greatly by socioeconomic factors. Risk factors for CAP in adult populations include: including age (>65 years <5 years), smoking, alcoholism, immunosuppressive conditions, and other chronic conditions such as COPD, cardiovascular disease, cerebrovascular disease, chronic liver or renal disease, diabetes mellitus and dementia (197).

Severe CAP accounts for 6% of UK intensive care unit (ICU) admissions with mortality estimated at 35%, representing half of all CAP deaths. Overall hospital mortality sits at 50%, with an increasing incidence due to an aging population and rising antimicrobial resistance this is set to rise (198).

Like sepsis, alternative treatments are need for the treatment of severe CAP.

Previous work, which has shown P4 peptide to be an effective treatment for pneumococcal pneumoniae in murine models as well as its capacity to augment phagocytosis in both peripheral blood neutrophil and alveolar macrophages from differing populations of healthy volunteers(162, 167). This study, for the first time, is moving on from animal models and healthy human subjects to looks at P4 augmentation of phagocytic responses in peripheral blood neutrophils and alveolar macrophages from patients admitted to the intensive care unit (ICU) with a primary diagnosis of CAP.

2. Opsonophagocytosis killing assay

The primary endpoint of this study was the opsonophagocytosis killing assay (OPK). This assay was developed by the Centre for Disease Control and Protection CDC and was used to measure killing by phagocytic cells of

intravenous IgG (IVIG) opsonised pneumococci with and without P4 stimulation (199).

3. Clinical data

In addition to the functional assays performed with patient cells, clinical data such as microbiology results, haematological and physiological measures were collected for each patient in order to see if there was any relationship between these measures and the cellular response to P4 peptide.

4. Cell surface marker and serum cytokines

Cell surface markers and serum cytokine levels were measured for each patient in order to see if there was any relationship between these measures and the cellular response to P4 peptide.

5. P4 stimulation

Ex vivo stimulation of peripheral blood neutrophils was performed to determine the effects of P4 peptide exposure on cell surface marker expression as well and the secretion of cytokines.

B. Methods and Materials:

1. Ethics Statement

We obtained national research ethics approval (12/NW/0730) for this study. Informed consent was obtained from patients.

2. Power calculations

It was calculated that 20 samples (α=0.05, β=0.05) would be required to demonstrate a 25.5% increase in bacterial killing based on previously published data using human alveolar macrophages (162).

3. Patients

Patients were recruited between March 2013 – March 2014 from Aintree University Hospital and The Royal Liverpool University Hospital.

Study inclusion criteria:

I. Adults (>18years)
II. Admitted to critical care with a diagnosis of severe community-acquired pneumonia (CAP) in accordance with British Thoracic Society Guidelines (136).

CAP was defined as: symptoms and signs consistent with an acute lower respiratory tract infection and new radiographic shadowing for which there was no other explanation. CAP was the primary reason for admission to ICU in all recruited patients.

Exclusion criteria:

I. Hospital admission within past 14 days
II. Pregnancy
III. immunocompromise (e.g. HIV Infection or chemotherapy).

4. Healthy Volunteers

Healthy volunteers were recruited to serve as technical controls and consented for both bronchoalveolar lavage and blood sampling.

Inclusion criteria:

I. Adults aged 18-65 years. Age chosen to minimise risk of bronchoscopy.
II. Fluent spoken English.

Exclusion criteria:

I. Asthma or pre-existing lung disease requiring regular immunoregulatory treatment or having any recent ill health
II. Cigarette smoking of greater than 10 pack years (20 cigarettes per day for 10 years)
III. Chronic illness
IV. Pregnancy

5. Sampling

All patients were recruited within 48 hours of admission to ICU and sampled within 7 days of recruitment. When research bronchoscopy was performed, blood samples were taken immediately prior to bronchoalveolar lavage. Venous blood samples were collected into 9 ml lithium heparin vacutainers.

Blood was transported from the hospital to the lab at room temperature in order to maintain optimal neutrophil viability. Research bronchoscopy was restricted to mechanically ventilated patients to avoid acute hypoxia in patients dependent on supportive oxygen therapy.

Healthy human volunteer bronchoscopy was undertaken using published technique (200). This technique was used with modifications for intubated critical care patients. Briefly, patients were given adequate intravenous sedation (usually a combination of propofol and alfentanil), 100% oxygen and monitored closely for the duration of the procedure. Experienced critical care physicians undertook bronchoscopy via the endotracheal tube to enable anticipation and prompt management of any complications. Bronchoalveolar lavage samples were transported from the hospital to the lab on ice.

6. Data collection

Relevant patient data was collected prospectively using an *a priori* case report form. This data included basic demographic details, clinical markers of severity, microbiology data and patient outcome measures.

7. Pneumococcal Stocks

Frozen pneumococcal stocks were prepared for use in OPK assays. Serotype 2, strain D39 was streaked on to 5% blood agar plates and incubated 16-18 hours at 37°C in gas jars.

The following day universal tubes containing 5 ml of BHI broth were inoculated with D39 from the agar plates and incubated statically for 16-18 hours. The culture was then centrifuged at 3000 g for 10 minutes, the

supernatant discarded and the pellet resuspended in 1 ml of 20% serum BHI broth and diluted to give a 10ml culture at OD_{500} of 0.7. This was incubated until the culture reached an OD_{500} of 1.2-1.4 (max 6 hour growth). This culture was then aliquoted into 2 ml screw top tubes and frozen at -80°C. Between 24 and 48 hours after freezing aliquots were thawed and the CFU determined by Miles and Misra.

8. Blood Cell Isolation

Cells were isolated from blood by dextran sedimentation followed by centrifugation over a Histopaque 1077 (Sigma-Aldrich, UK).

Blood was transferred from vacutainers (3 x 9 ml lithium heparin) into a 50 ml centrifuge tube containing 50 µl of heparin sodium salt (5 units/µl), (Sigma-Aldrich, UK). The blood was then mixed 2:1 with 6% dextran (MW >500,000, Fisher, UK) and left for 20-30 minutes at room temperature for the red blood cells to sediment (see Figure 54, section A).

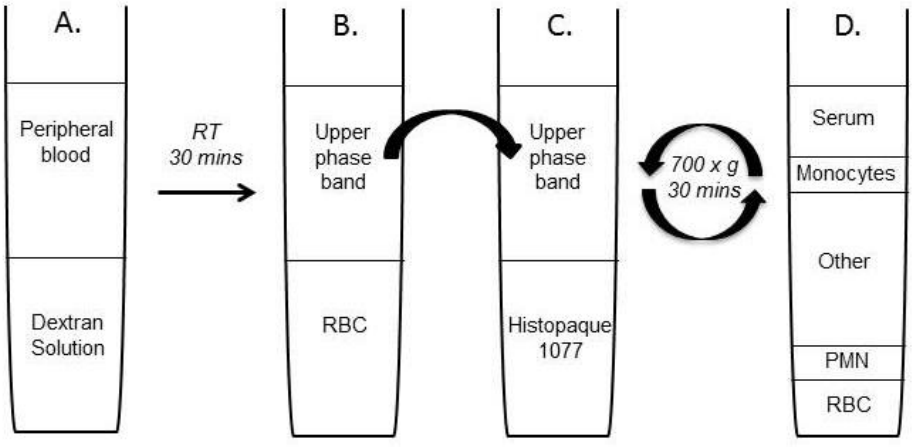


Figure 54 Blood cell isolation – dextran sedimentation and density centrifugation

After 20-30 minutes there should be two distinct phases; a dark red lower phase containing the red blood cells and a pale pink upper phase rich in leukocytes (see Figure 54 section B).

The upper phase was collected and layered over 7 ml of Histopaque 1077 in a 15 ml centrifuge tube (see Figure 54, section C), 2 -3 tubes were needed per patient. Samples were then centrifuging at room temperature, 700g for 30 minutes with no brake.The neutrophil (PMNs) and monocyte fractions were collected into separate 50 ml centrifuge and washed in Hank's Buffered Salt Solution without Magnesium or Calcium (HBSS-/-), residual red blood cells were lysed with BD Pharm Lyse (BD Biosciences, USA) as per the manufacturer's instructions. Cells were then counted with a haemocytometer and viability assessed by trypan blue exclusion.

9. Alveolar Macrophage Isolation

BAL samples were passed through a 100 µm cell strainer (BD Bioscience, Germany) before centrifuging at 400g, 4°C. BAL sample supernatants were aspirated and aliquoted for storage at -70°C. Cell pellets were washed and counted before resuspending in RPMI 1640 supplemented with 10% FBS, penicillin and streptomycin. Cells were seeded into flat bottom 96 well plates at a density of 1×10^5 alveolar macrophages per well. Cells were incubated at 37°C, 5% CO_2 for two hours to allow alveolar macrophages to adhere before washing with PBS three times to remove non-adherent cells and antibiotic containing media.

10. Opsonisation of pneumococci

Pneumococcal stocks were thawed, washed twice by centrifuging at 13,000 rpm, discarding the supernatant and resuspending in +/+ HBSS, 5% FBS to give a final concentration of 5 x 10^4 CFU/ml (alveolar macrophage assays used 5 x 10^5 CFU/ml). IVIG (Gamunex-C, Grifols, Spain) was diluted 1:1 in +/+ HBSS, 5% FBS and then mixed with the diluted pneumococci 1:1 for opsonisation - 20 minutes, 37°C at 100rpm. The final concentration of IVIG in the assay is 1:16 which equates to 0.5 µg of protein. Un-opsonised controls used incubated as above in +/+ HBSS, 5% FBS at the same dilution.

11. Opsonophagocytosis Assay - neutrophils

Isolated neutrophils were seeded into 96 well U bottomed plates at a density of 5 × 10^4 neutrophils per well were incubated with 5 × 10^2 opsonised *S. pneumoniae* (20 µl per well of the pneumococci and IVIG mix from Opsonisation of pneumococci), 10 µl baby rabbit complement (Mast Group, UK), and P4 solution (10 µg/well) for 45 minutes at 37°C, 175rpm. CFU were determined following incubation by Miles and Misra. Wells not being treated with P4 received DEPC-treated water. Wells containing no neutrophils, non-opsonised pneumococci and heat-inactivated complement were used as further controls. Killing index was calculated as 1 – dose well/OPA well (dose well - opsonised *S. pneumoniae* and complement, OPA well - phagocytes, opsonised *S. pneumoniae* and complement +/- P4 peptide)

12. Opsonophagocytosis Assay – alveolar macrophages

Alveolar macrophages were seeded into 96 well flat bottomed plates (as detailed in Alveolar Macrophage Isolation) at a density of 1 x 10^5/well. After washing 5 × 10^3 opsonised *S. pneumoniae* (20 µl per well of the pneumococci and IVIG mix from Opsonisation of pneumococci) were added to wells along with 10 µl of 3-4 week baby rabbit complement and P4 solution (20 µg/well) for 45 minutes at 37°C, 100rpm. CFU were determined following incubation by Miles and Misra. Control wells received DEPC-treated water. Wells containing no macrophages, non-opsonised pneumococci and heat-inactivated complement were used as further controls. Results of OPK assays were expressed as percentage killing index.

This was calculated as:

$$100 - \left(\frac{OPK\ Well}{Dose\ Well}\ x100\right)$$

Dose well - opsonised *S. pneumoniae* and complement

OPK well - phagocytes, opsonised *S. pneumoniae* and complement +/- P4 peptide.

13. P4 Stimulation

Isolated neutrophils were resuspended in RPMI 1640 (Life Technologies, USA) supplemented with 10% FBS (Sigma-Aldrich, UK) and seeded into 96 well plates at a density of 2 x 10^5 cells per well. P4 peptide was added to wells at a concentration of 20 µg/well. Control wells were made up to equal volume with DEPC-treated water. Cells were incubated at 37°C, 30rpm for

45 minutes and 6 hours at which point cell were centrifuged at 300g, supernatants collected (stored at -70°C) and cell stained for cell surface markers following the procedure detailed below.

14. Flow-cytometry

Cell suspensions were stained for 20 minutes at 4°C (detailed in section II.B.4.b. The following antibodies were used:

Ebioscience, UK

- CD64 (FcγRI, 10.0-FITC)
- CD32 (FcγRII, 6C4-APC)
- CD16 (FcγRIII, CB16-PeCy7)
- CD11b (CR3/Mac-1, CBRM1/5-PE)

Biolegend, USA

- CD88 (C5aR, S5/1-PeCy7)
- CD35 (CR1, E11-FITC)
- CD66b (CEACAM1, G10F5-PE)
- CD181 (IL-8RA, 8F1/CXCR1-FITC)

All cells were incubated with Trustain FcX Fc receptor blocking solution (Biolegend, USA) prior to staining with the exception those being stained with for CD64, CD32 and CD16. Isotype controls were used to exclude non specific binding. Acquisition was carried out using an Accuri C6 (BD Biosciences, USA) flow cytometer, and analysis was performed using FlowJo 8.7 for Macintosh (Tree Star).

15. Cytokines

Serum was collected from each patient in 7.5 ml S-Monovette Z serum tubes (Sarstedt, Germany), tubes were centrifuged at 2000 g for 10 minutes and the serum aliquoted for storage at -70°C.

IL-6, IL-8 and IL-10 levels were measured using Ready-SET-Go ELISAs (Ebioscience, UK) performed as per manufacturer's instructions. Plates were read with a FLUOStar Omega plate reader and analysised with the MARS Data Analysis interface (BMG Labtech, Germany).

16. Statistical Analysis

Statistical analysis was performed with Prism 5 (Graphpad Software, USA), individual tests are noted in figure legends.

C. Results

1. Healthy Controls

Five healthy controls were recruited and OPK assays performed on their neutrophils throughout the study to help ensure all reagents were functioning as expected for the length of the study.

The neutrophil OPK showed a statistically significant increase in killing index with P4 treatment (p=0.04), mean killing was 11% at baseline and increased to 28% with P4 treatment (Figure 55).

The alveolar macrophage OPK showed an increase in mean killing from 24% at baseline to 52% with P4 treatment however was no statistically significant (p=0.1) (Figure 55).

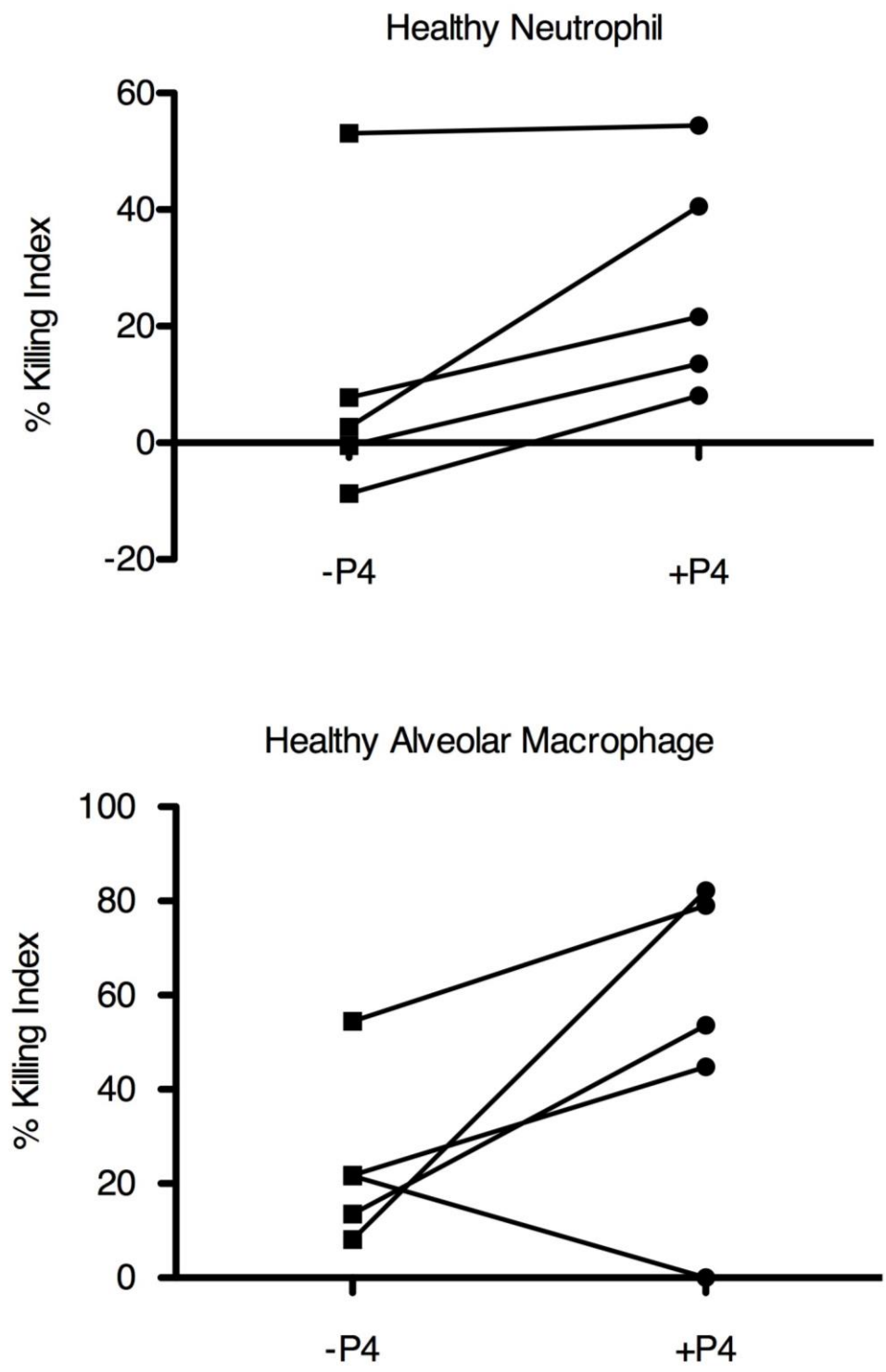


Figure 55 OPKs from blood neutrophils and alveolar macrophages of healthy volunteers

Killing index of untreated and P4 treated peripheral blood neutrophils (top) and alveolar macrophages (bottom) from health controls. Neutrophils p=0.04, alveolar macrophages p=0.1 paired t-test.

2. Patient Characteristics

25 patients with community-acquired pneumonia (as defined by British Thoracic Society criteria [2]) were recruited into the study. All patients had severe pneumonia requiring admission to critical care for organ support and met the criteria for sepsis (see introduction). Table 16 depicts characteristics for 23 patients (two patients were excluded due to technical failure in testing) in our cohort.

Samples were taken at a mean of 52 hours from admission (range 13-110 hours) and processing began within one hour of sample collection. Fifteen (65%) patients were male, median age 54 (IQR 44-72) and median Acute Physiology and Chronic Health Evaluation II (APACHE II – disease severity score for ICU calculated within 24 hours of admission) score 15, IQR 11-24 (Table 16).

The was no relationship between APACHE II score and response to P4 peptide in the OPK assay observed in our cohort although there was a trend towards a higher Sequential Organ Failure Assessment score (SOFA – scoring system used to track organ function in ICU, ranges from 0-24) in patients that did not respond to P4 in the OPK assay (mean SOFA score responders =5, mean SOFA score non responders = 8) (Figure 60).

The 28 day survival for our cohort was 19/23 (17% mortality), of the four patients who died within 28 days, three died in the ICU. Ten patients had a causative organism identified (three blood culture positive). The most commonly isolated organism was *Streptococcus pneumoniae* (n=3, 1 blood culture, 2 urinary antigen) (Table 16).

Major co-morbidities included: chronic obstructive pulmonary disease (8/23), ischaemic heart disease (6/23) and diabetes mellitus (4/23).

Antimicrobial therapy for the majority of patients was a combination of either Piperacillin/Tazobactam (Tazocin) or benzylpenicillin combined with clarithromycin (Table 16).

Table 16 – Patient Characteristics – Orange = Responders, Blue = Non responders

ID	Age	Microbiology	APACHE II	Antimicrobial therapy	SOFA (Sampling)	Co-morbidities	28 Day Survival
A	83	*E. coli*	24	PIP/TAZ, CLAR	10	Asthma, IHD, AF	YES
B	30	*S. milleri*	11	PIP/TAZ, CLAR	7	Trisomy 21	YES
C	42	Neg	8	CLAR, BPC	1	SLE, DVT	YES
D	37	*H. influenza*	8	Co-amoxiclav, CLAR	0	IVDU, Hepatitis C, DVT	YES
E	49	Neg	26	BPC, CLAR	6	COPD, IBD	YES
F	54	*Legionella*	15	CLAR	2	IBD	NO
G	73	*Influenza A*	24	PIP/TAZ, Tamiflu, CLAR	8	HTN, Breast cancer (treated)	YES
H	47	Neg	14	CLAR, BPC, Tamiflu	2	T2DM, IHD, CKD3	YES
I	50	*S. pneumoniae*	18	Not completed	5	Alcoholism	YES
J	78	*S. pneumoniae*	14	CLAR, BPC	9	Nil significant	YES
K	61	Neg	9	PIP/TAZ, CLAR	3	COPD, RA	NO
L	72	Neg	22	Ciprofloxacin, PIP/TAZ	9	T2DM, mild dementia	YES
M	60	Neg	15	CLAR, BPC	2	COPD, AS	YES

ID	Age	Microbiology	APACHE II	Antimicrobial therapy	SOFA (Sampling)	Co-morbidities	28 Day
N	39	Neg	13	CLAR, BPC	5	Asthma, T2DM, HTN	YES
O	31	*Parainfluenza* type 1	14	PIP/TAZ, Teicoplanin	4	Quadriplegic	YES
P	69	Neg	25	CLAR, BPC	12	IHD, Peripheral neuropathy	YES
Q	75	Neg	25	BPC, CLAR	9	AS, HTN, Stroke	NO
R	44	*S. pneumoniae*	8	PIP/TAZ, CLAR	3	COPD, IVDU, Asthma	YES
S	72	*S. pneumoniae*	24	BPC	10	Asthma, HTN Asbestosis, IHD	YES
T	48	Neg	25	CLAR, BPC	6	Paraplegic	YES
U	75	Neg	6	CLAR Vancomycin	10	COPD, HTN, Laryngeal cancer	YES
V	69	*E. coli*	13	PIP/TAZ, CLAR,	14	COPD, ALD (Child C)	NO
W	49	Neg	2	CLAR, BPC	3	Sleep Apnoea, T2DM, HTN	YES

APACHE II - Acute physiology and chronic health evaluation II
SOFA – Sequential organ failure assessment

Antimicrobial therapy:
BPC – Benzylpenicillin
CLAR – Clarithromycin
PIP/TAZ –Tazocin

Co-morbidities:
AF – Atrial fibrillation
ALD – Alcoholic liver disease
AS – Aortic stenosis
CKD – Chronic kidney disease
COPD – Chronic obstructive pulmonary disease
DVT – Deep vein thrombosis
HTN – Hypertension
IBD – Inflammatory bowel disease
IHD – Ischaemic heart disease
IVDU – Intravenous drug user
RA – Rheumatoid arthritis
SLE – Systemic lupus erythematosus
T2DM – Type 2 Diabetes mellitus

3. Opsonophagocytosis Assays

Blood neutrophil OPKs were completed in 23/25 patients (2 excluded due to technical failure). Fourteen (60%) samples responded to P4 peptide stimulation (fold increase > 1) (Figure 56).

Treatment with P4 peptide compared to carrier control led to significantly improved *ex vivo* neutrophil phagocytic killing of pneumococci with a mean killing index of 32% vs. 20% (mean difference 11.2%, C.I. 4.4-18.0, p=0.002) (Figure 57).

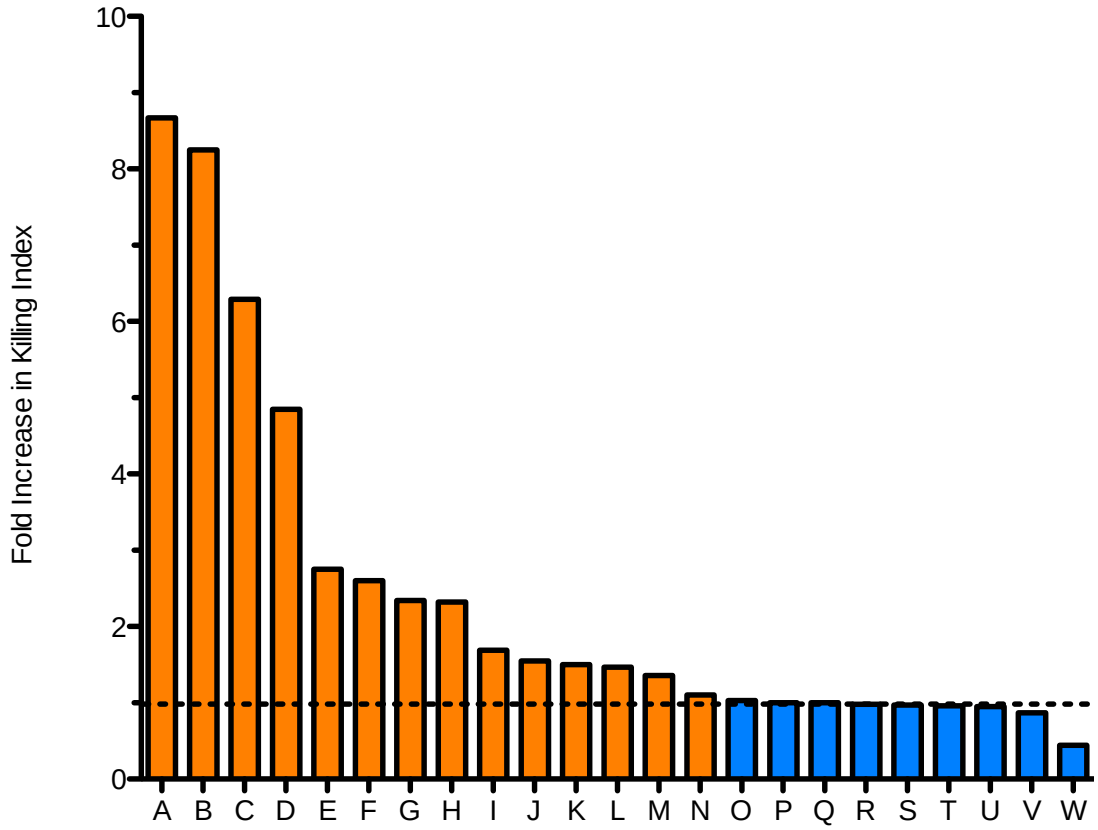


Figure 56 – Fold increase in killing index of peripheral blood neutrophils treated with P4 peptide

Fold increase in killing index of P4 treated neutrophils when compared to untreated neutrophils for each of the 23 ICU patients. 56% (14/23) patients showed an increase in killing when treated with P4 peptide. Dotted line indicates a fold increase of 1, patients with a fold increase of 1 or less are considered non responders and are highlighted in blue (O-W), responders are highlighted in orange (A-N). Letters on the X axis correspond with the ID column in Table 16.

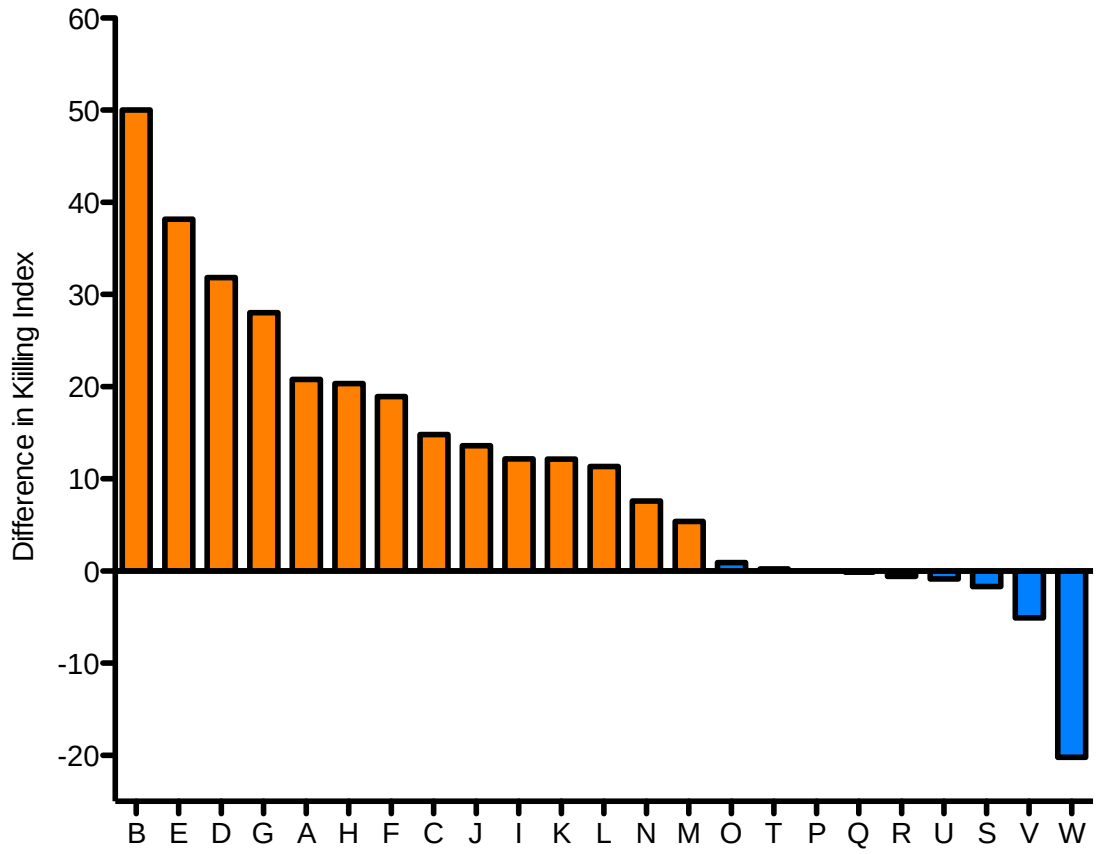


Figure 57 – Difference in killing index between P4 treated peripheral blood neutrophils and untreated neutrophils

Difference in killing index between P4 treated and untreated peripheral blood neutrophils for each of the 23 ICU patients recruited (P4 killing index minus untreated killing index in each patient recovered neutrophil sample). 56% (14/23) patients showed an increase in killing when exposed to P4 peptide. Letter on the X axis correspond with the ID column in Table 16. Non responders and are highlighted in blue (O-W), responders are highlighted in orange (A-N). The cut-off for a response was set at a difference in killing index of 5 or more.

Patients were divided into responders and non responders using the cut off of an increase in killing index of 5% or more. Non responders had a mean baseline phagocytosis (OPK killing index with carrier control) of 30% this remained fairly static with P4 treatment killing index at 27%. The mean baseline phagocytosis for responders was 14%, with P4 treatment this increased significantly to 34% ($p<0.0001$ paired t test) (Figure 58). The difference between the baseline phagocytosis for responders and non responders was approaching significance ($p=0.08$, Mann Whitney). Figure 59 shows the killing index before and after P4 treatment for each individual patient.

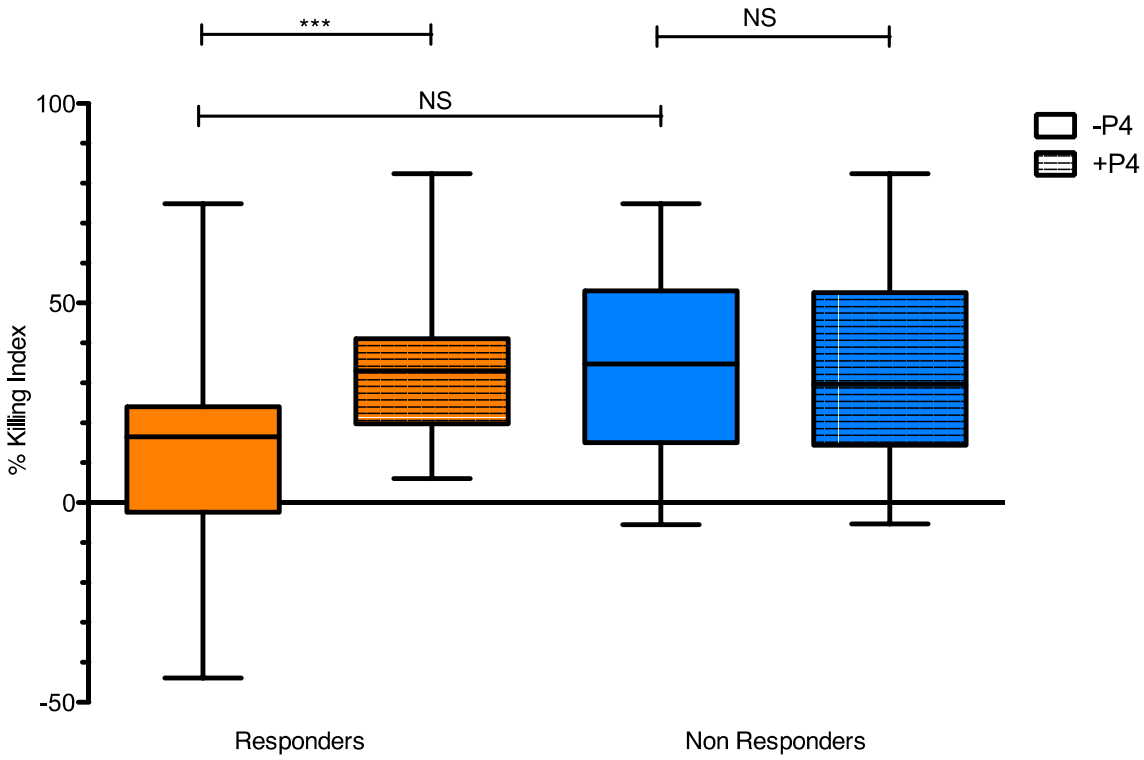


Figure 58 Killing index for peripheral blood neutrophils treated with P4 peptide and untreated neutrophils

Killing index of untreated and P4 treated peripheral blood neutrophils. Patients are divided into responders in orange (increase in killing index of 5 or more with P4 treatment, n=13) and non responders in blue (increase in killing index of less than 5, n=10). Responders showed a significant increase in killing index with P4 treatment ($p<0.0001$, paired t test). P4 treated groups are indicated by crosshatching on bars.

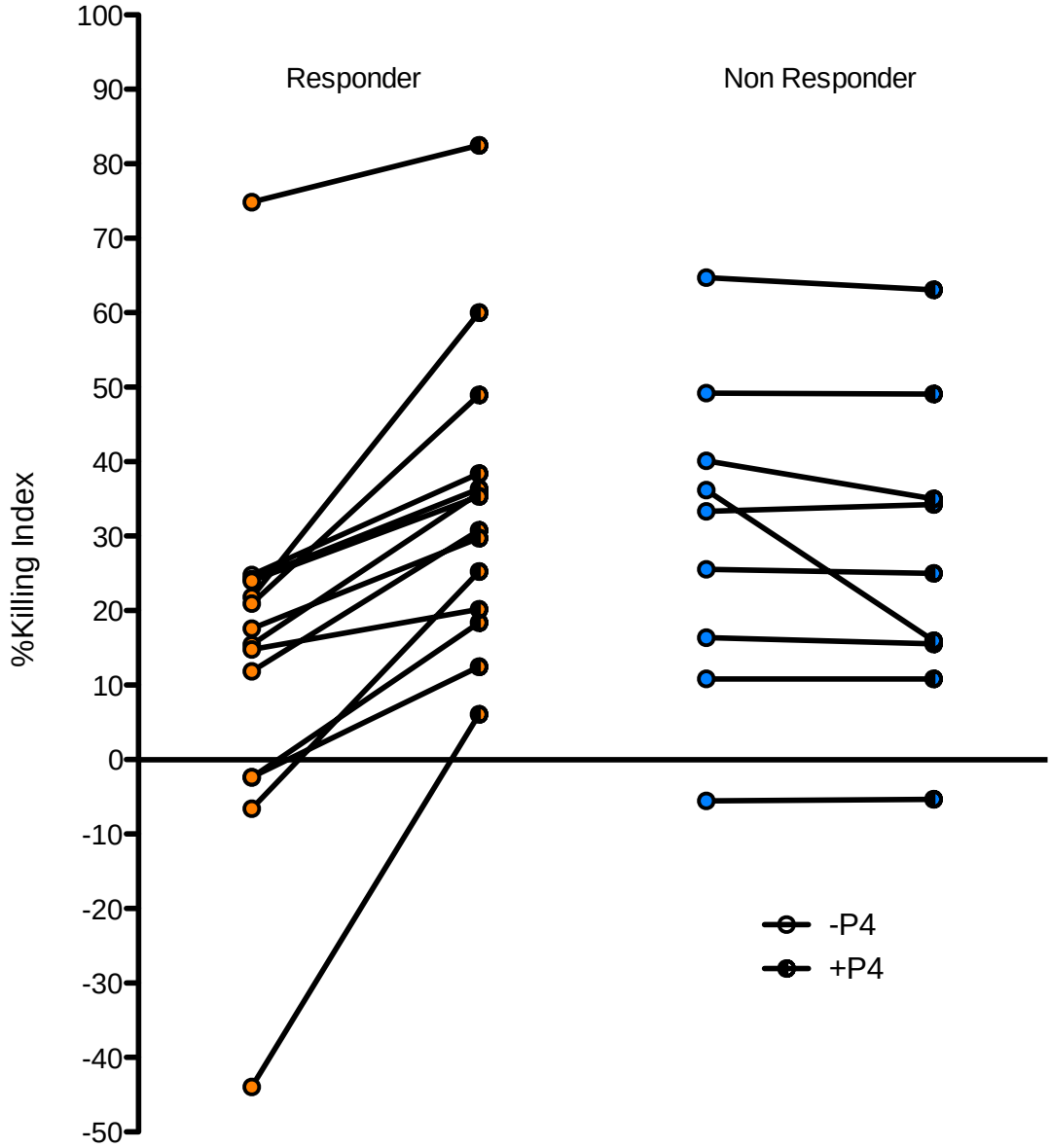


Figure 59 Killing index for peripheral blood and untreated neutrophils for individual patients neutrophils treated with P4 peptide

Killing index of untreated and P4 treated peripheral blood neutrophils. Patients are divided into responders in orange (increase in killing index of 5 or more with P4 treatment, n=13) and non responders in blue (increase in killing index of less than 5, n=10). When all 23 patients are taken into account treatment with P4 peptide resulted in a significant increase in killing index (p=0.004), Wilcoxon signed rank test.

4. Patient Characteristics – Clinical Parameters

The APACHE II and SOFA scores for patients and the predicted mortality associated with scores. Both the APACHE II and SOFA score are measures used in ICUs to assess disease severity. The APACHE II score is calculated at the time of admission (or within 24 hours) and is based on 12 physiological parameters as well as patient age. Based on the APACHE II score there was no significant difference in the disease severity for the two groups at admission (Figure 60, top panel).

The SOFA score is calculated sequentially to monitor organ failure and is based on 6 scores assessing function in the respiratory, cardiovascular, hepatic, coagulation, renal and neurological systems. The non responder group showed a trend towards higher SOFA scores than responders which was approaching significance (p=0.06, t-test) (Figure 60, bottom panel).

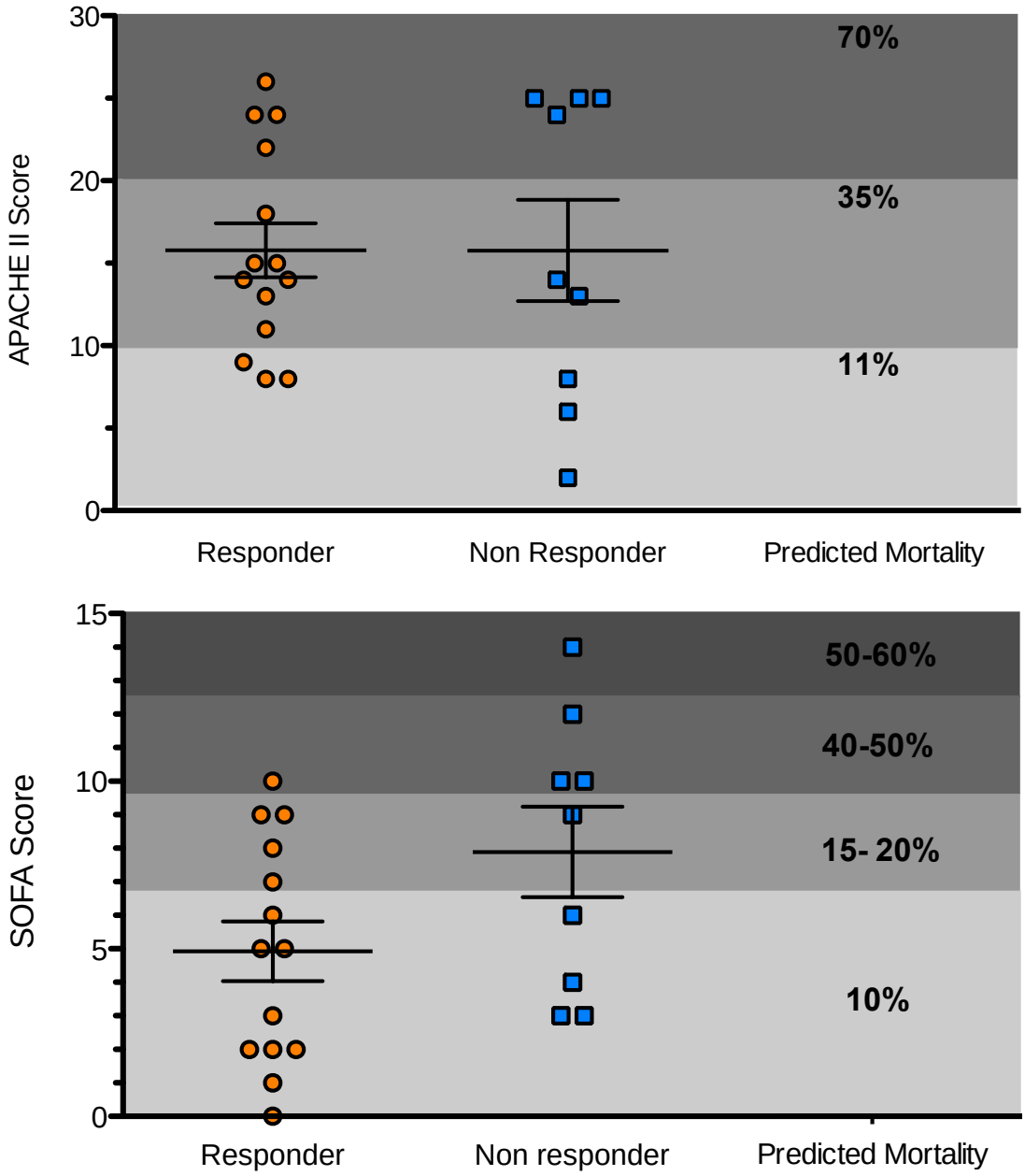


Figure 60 APACHE II and time of sampling SOFA scores
APACHE II and SOFA scores for CAP patients recruited to study (n=14 responders - orange circles, n=9 non responders - blue squares). Patients are divided into responders and non responders depending on the response of their peripheral blood neutrophils to P4 peptide in the OPK. Top: The acute physiology and chronic health evaluation II score (APACHE II) was calculated within 24 hours of admission to ICU. There was no significant difference in APACHE II score between groups (t-test). Bottom: The sequential organ failure assessment was calculated at the time of sampling. Non-responders had a higher SOFA score than responders (mean responders =5, mean non responders = 8) these values were approaching significance (p=0.06, t-test).

The time from patient admission to sample collection was kept to a minimum but due to logistical reasons (such as admission at night or delays in acquiring consent) it was not always possible to acquire samples promptly. Sampling times ranged from 13 to 110 hours (mean 52 hours). The sampling time did not correlate with the patient SOFA score in our cohort (Figure 61, Top). The level of response to P4 peptide treatment also did not show a relationship with the time from sampling (Figure 61, Bottom).

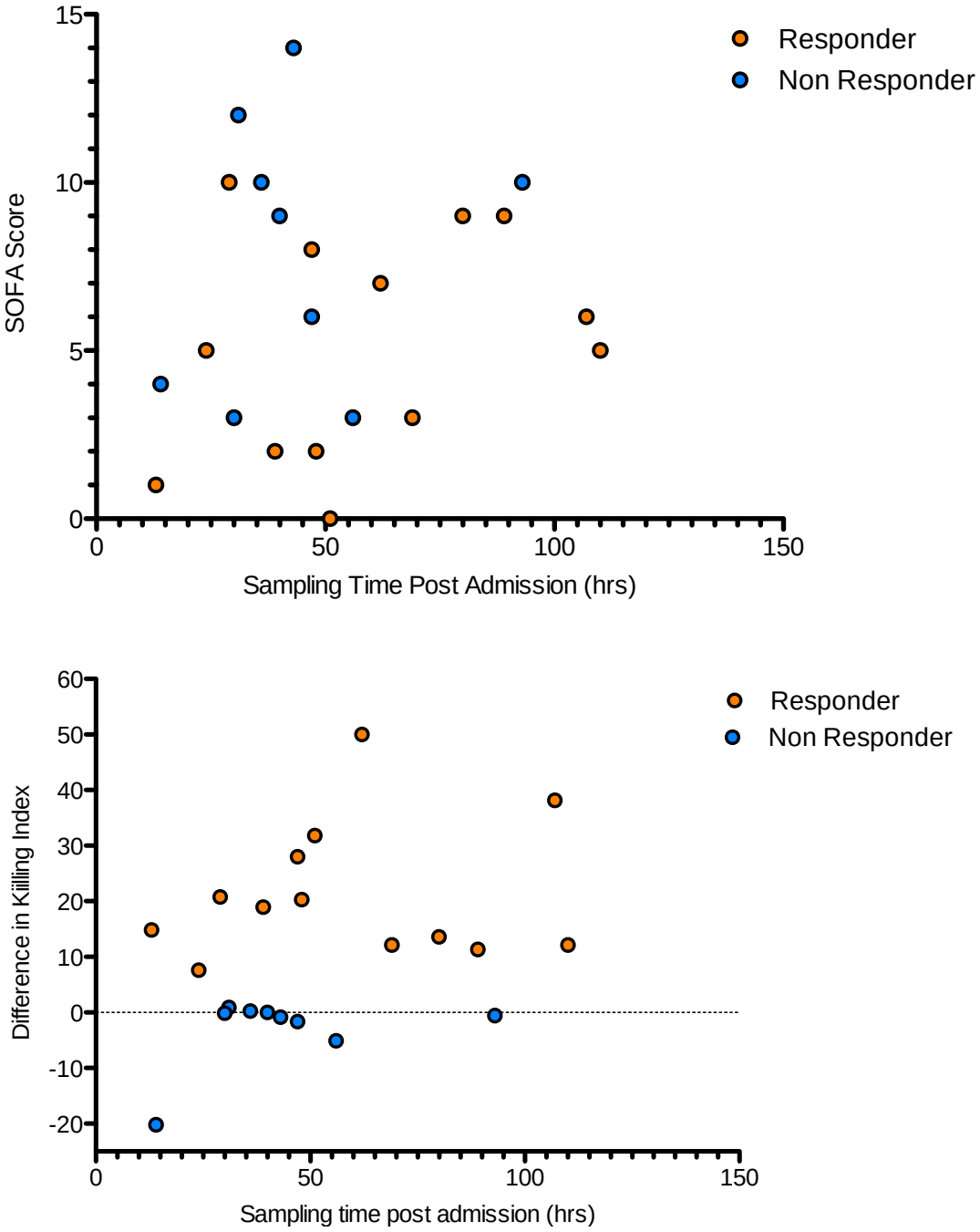


Figure 61 Effect of sampling time
Effect of sampling time. Top: Patient SOFA score vs. time of sampling post admission – there was no relationship observed between the patient SOFA score and the time from admission to sampling in this cohort. Bottom: Difference in killing index vs. time of sampling post admission – there was no relationship observed between the difference in killing index and the time from admission to sampling in this cohort.

5. Patient Characteristics – Neutrophils

White blood cell (WBC) and neutrophil counts were performed by the hospital haematology service. When divided into responders and non-responders (Figure 62) there was no difference between the two groups in neutrophil or WBC count (p=0.1 and p=0.1 respectively).

It was shown however that there was a weak (R squared value = 0.3) yet statistically significant (p=0.001) correlation between neutrophil count and the fold increase in killing with P4 peptide treatment in the OPK (Figure 63).

It was noted that the patients with the highest neutrophil counts tended to have some of the lowest baseline phagocytosis values, although not statistically significant in this study (p=0.057).

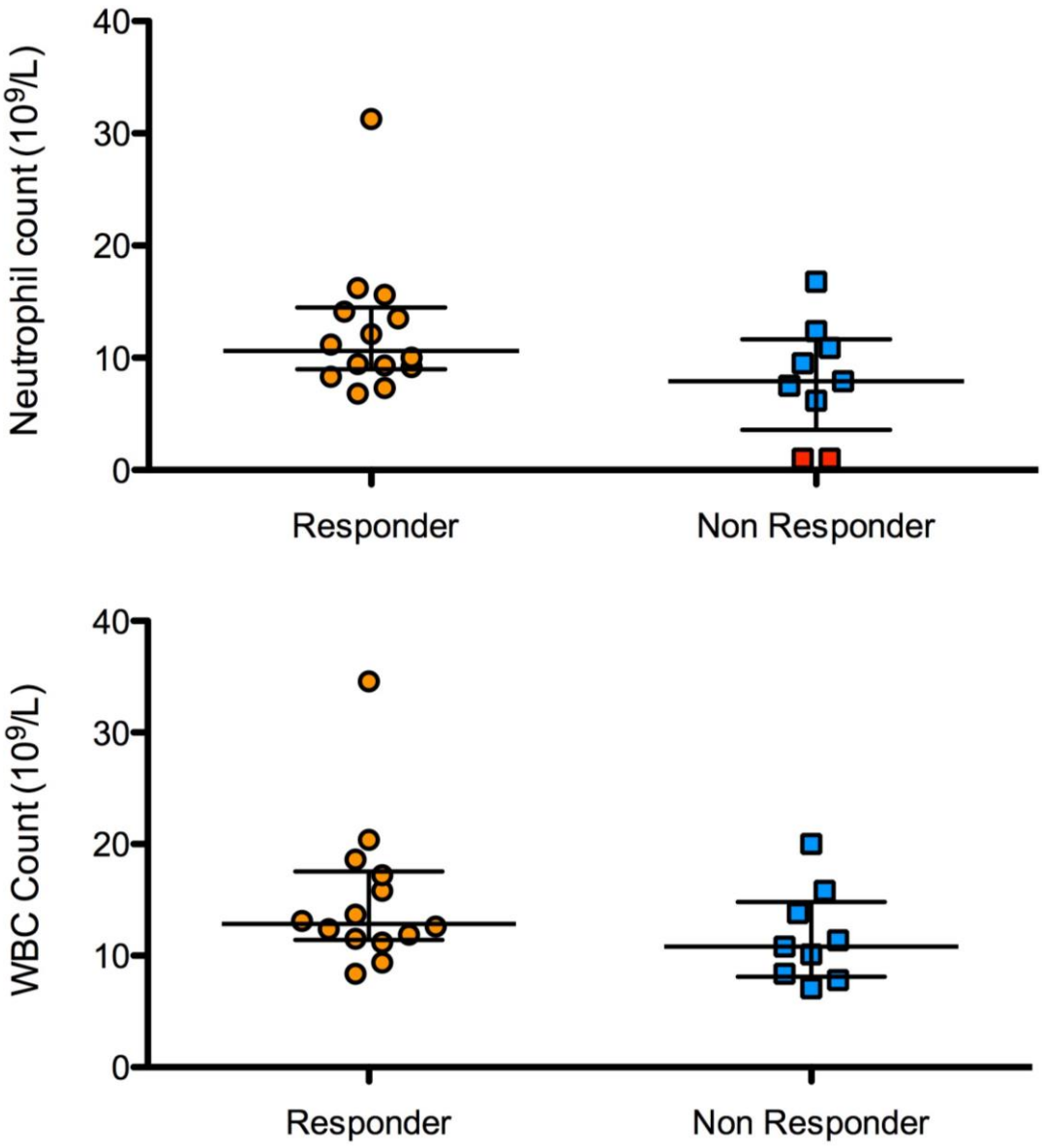


Figure 62 Patient neutrophil and WBC count
Neutrophil counts were provided for each patient by the hospital haematology laboratory. Top: Neutrophil count, bottom: white blood cell count (WBC) plotted as median and IQR. There was no statistical difference in neutrophil count between responders and non responders: neutrophil p=0.1, WBC p=0.1 Mann Whitney test. Patients marked in red had a neutrophil count below the detectable level for the automated counter – these patients have been recorded as having a neutrophil count of $1x10^9$/L for statistical purposes.

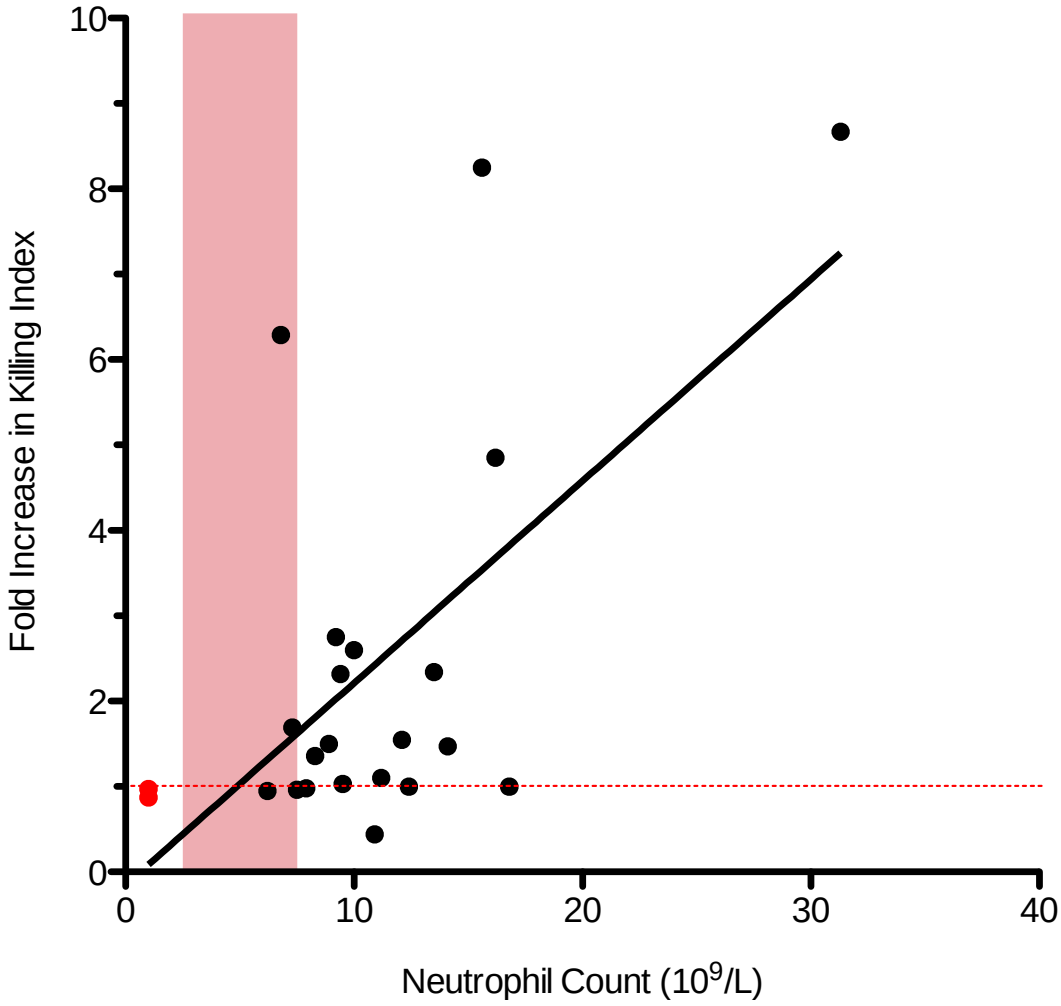


Figure 63 Neutrophil count vs fold increase in killing index
Neutrophil count plotted against fold increase in neutrophil killing. Dotted red line indicates a fold increase of 1, pink band indicates the normal/health range for neutrophil count. R squared value = 0.3, p=0.0019. Patients marked in red had a neutrophil count below the detectable level for the automated counter – these patients have been recorded as having a neutrophil count of $1x10^9$/L for statistical purposes.

6. Patient Characteristics - Cytokines

Serum cytokine levels for each patient were measured by ELISA. The detection threshold for the ELISA kits used was 2 pg/ml, where patients had cytokine levels lower than the threshold of detection they were recorded as 1 pg/ml for statistical purposes. Patients were divided into responders and non responders for the analysis of serum cytokines.

Responders had lower levels of IL-8 (median 11 pg/ml vs 88 pg/ml) and IL-10 (median 1 pg/ml vs 8.62 pg/ml) than non responders (p=0.03 and p=0.01 respectively, Mann Whitney) (Figure 64).

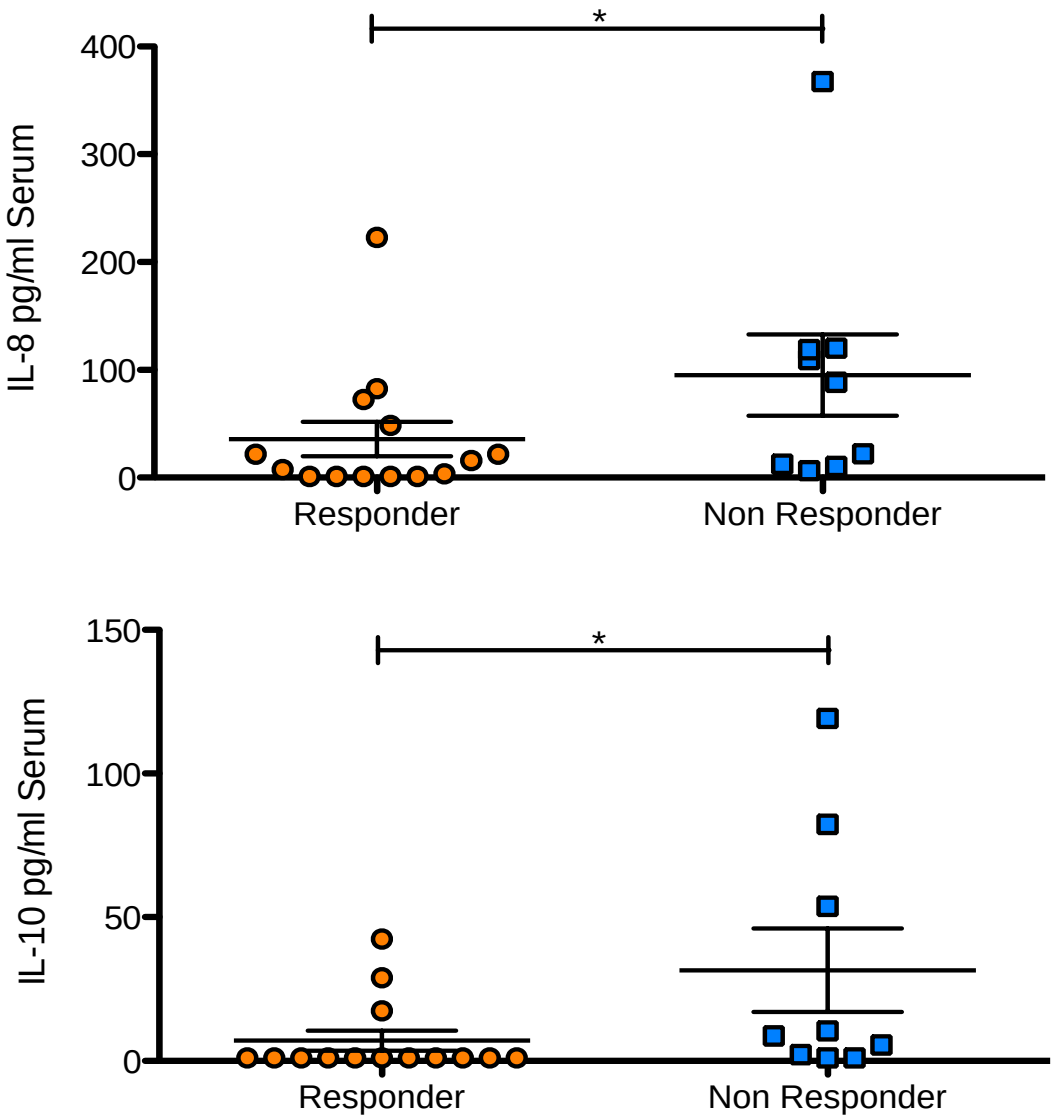


Figure 64. Patient serum cytokines – IL-8 and IL-10
Serum cytokine levels for each of the 23 ICU patients recruited divided in respoders (orange circle) and non-responders (blue squares). * = p<0.05 Mann Whitney test. Top- Serum IL-8 levels for individual patients, 5/23 patients had IL-8 levels below the detectable level (2 pg/ml) and were recorded as 1 pg/ml for statistical purposes. Median IL-8 level for responders was 11 pg/ml vs 88 pg/ml for non-responders (p=0.03, Mann Whitney test). Bottom- Serum IL-10 levels for individual patients, 13/23 patients had IL-10 levels below the dtectable level (2 pg/ml) and were recorded as 1 pg/ml for statistical purposes. Median IL-10 level for responders was 1 pg/ml (11/14 < 2 pg/ml) vs 8.62 pg/ml for non-responders (p=0.01, Mann Whitney test).

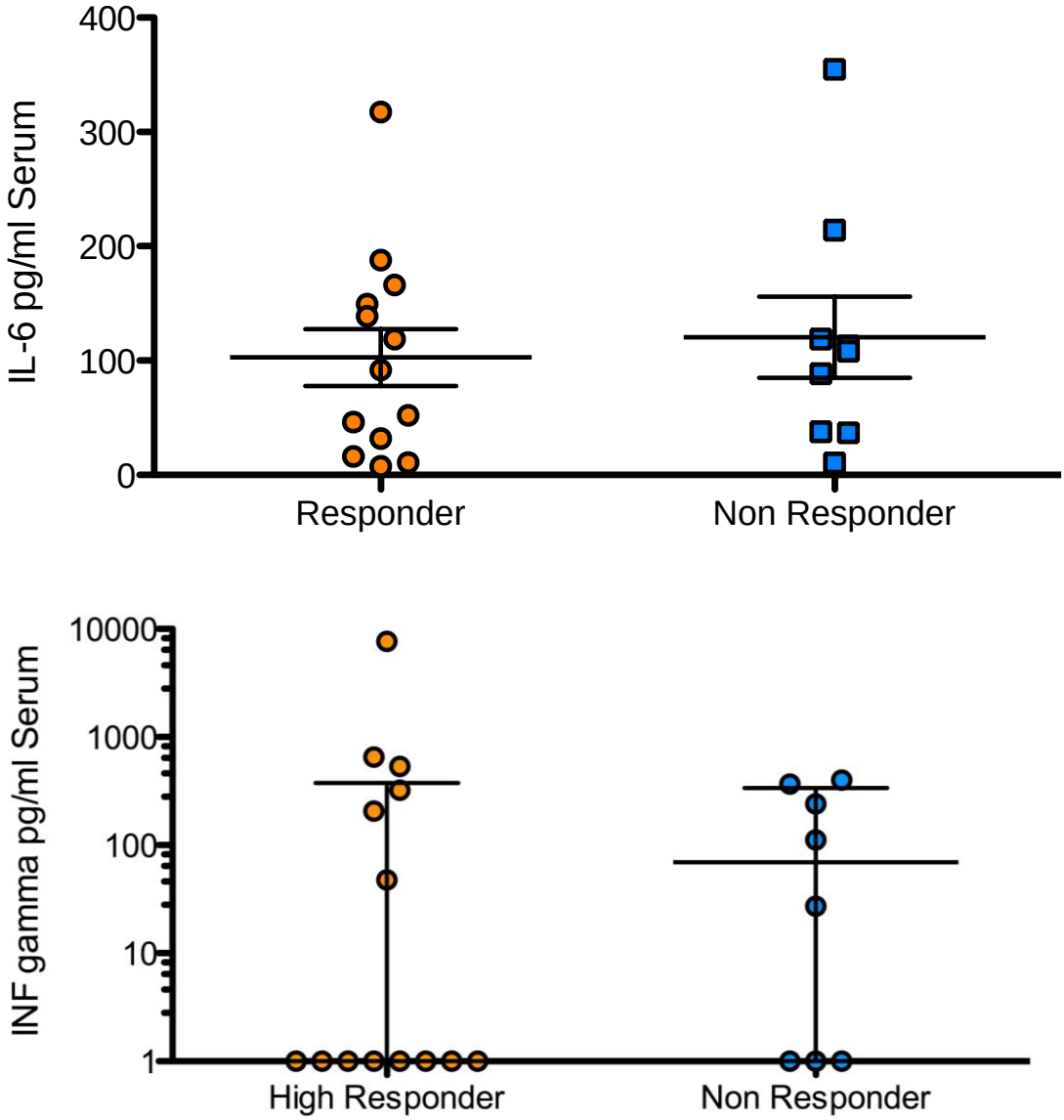


Figure 65 Patient serum cytokines IL-6 and INF-γ
Serum cytokine levels for each of the 23 ICU patients recruited divided in respoders (orange circle) and non-responders (blue squares). Top: Serum IL-6 levels for individual patients – there was no statistical difference between patient levels for responders vs non responders (median 92 vs 108 pg/ml, *p*=0.8, Mann Whitney test). Bottom: Serum INF-γ levels for individual patients – there was no statistical difference between patient levels for responders vs non responders (median 1.0 vs 70 pg/ml, *p*=0.7, Mann Whitney test).

Figure 66 shows there was a weak yet significant negative correlation between serum IL-8 level and neutrophil count (R squared value = 0.2, *p*=0.02). IL-8 was also observed to have a positive relationship with the baseline level of phagocytosis, the higher the IL-8 the higher the baseline killing, although not significant for the cohort as whole (*p*=0.05) this relationship was significant when only the non responders were taken into account (R squared value =0.4, *p*=0.04).

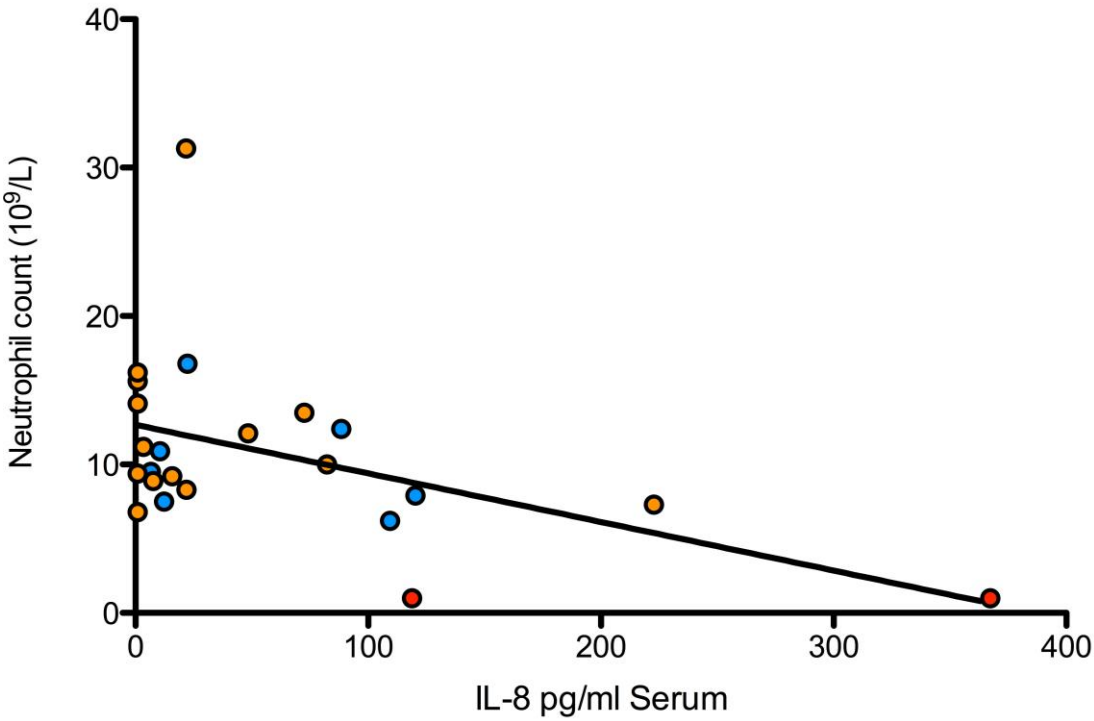


Figure 66 Patient neutrophil count vs. serum IL-8

Neutrophil count for each patient was plotted against their serum IL-8 level. R squared value = 0.2, *p*=0.02. Patients marked in red had a neutrophil count below the detectable level for the automated counter – these patients have been recorded as having a neutrophil count of $1x10^9$/L for statistical purposes – both were non responders. 7/23 patients had IL-8 levels below the detectable level (2 pg/ml) and were recorded as 1 pg/ml for statistical purposes.

7. FcγR Expression

The expression of CD64, CD32 and CD16 (FcγR I, II and III) on peripheral blood neutrophils was measured before as a baseline measurement and after a 45 minute stimulation with P4 peptide. There was no difference in baseline level of expression between responder and non responder group for any of the three receptors (Figure 67).

CD32 expression on peripheral blood neutrophils of responders was shown to decrease by 46% from its baseline value when incubated with P4 peptide ($p<0.01$, Dunn's multiple comparison following Friedmans test), there was no significant change in CD32 expression in the non responder group (Figure 68).

There was also no difference in observed in CD64 or CD16 after incubation with P4 peptide (Figure 69).

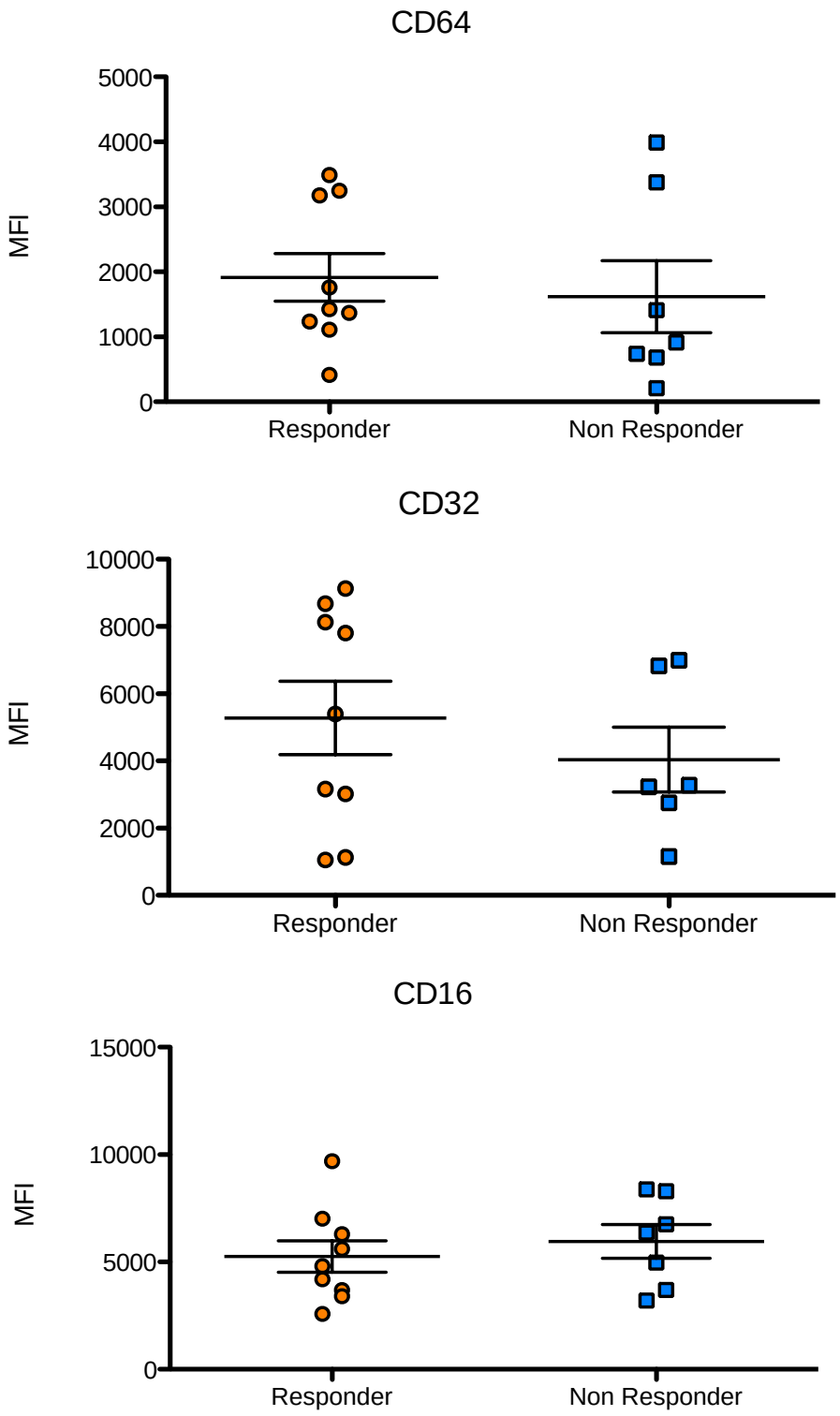


Figure 67 Fc gamma receptor expression - CD64 (FcγRI), CD32 (FcγRIIA/B)and CD16 (FcγRIII)

Fc gamma receptor expression on neutrophils from patients (n=9 responders, n=7 non responders, n=7 patients lacked sufficient neutrophil numbers for testing or the sample was received too late for testing) using flow cytometry. There was no significant difference between the two groups in the expression level of CD64 *p*=0.4, CD32 *p*=0.6 or CD16 *p*=0.4, - Mann Whitney test.

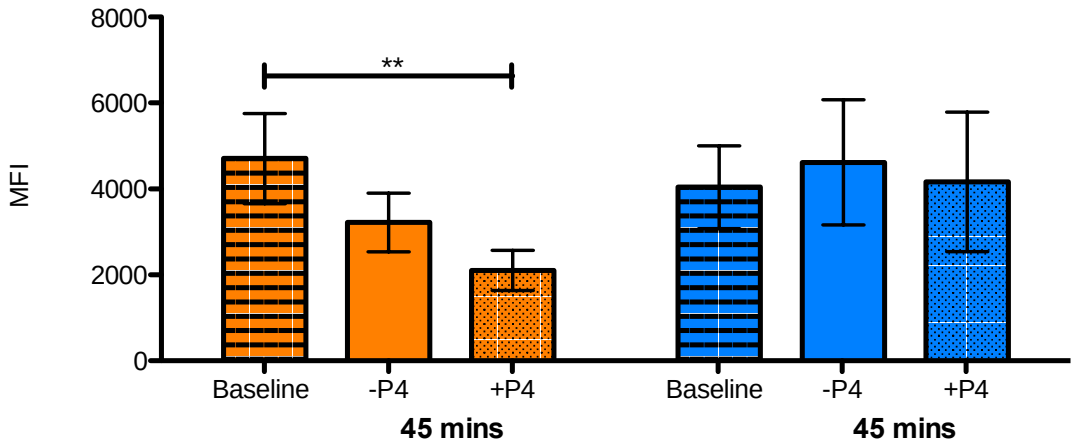


Figure 68 Effect of P4 on expression of CD32 on peripheral blood neutrophils
Peripheral blood neutrophils were isolated from CAP patients before incubating for 45 minutes in the presence of the P4 peptide. Cells were washed, stained with anti-CD32 (FcγRIIA/B) antibodies and expression levels determined by flow cytometry. Patients are divided into responders (orange, n=8) and non responders (blue, n=7). The responders showed a statistically significant drop from their baseline CD32 expression after P4 treatment (responders p =0.03, non responders p=0.1, Friedman test, **= p<0.01 Dunn's Multiple Comparison test). The responders saw a mean drop from baseline of 46%. Untreated neutrophils showed a drop from baseline of 27%.

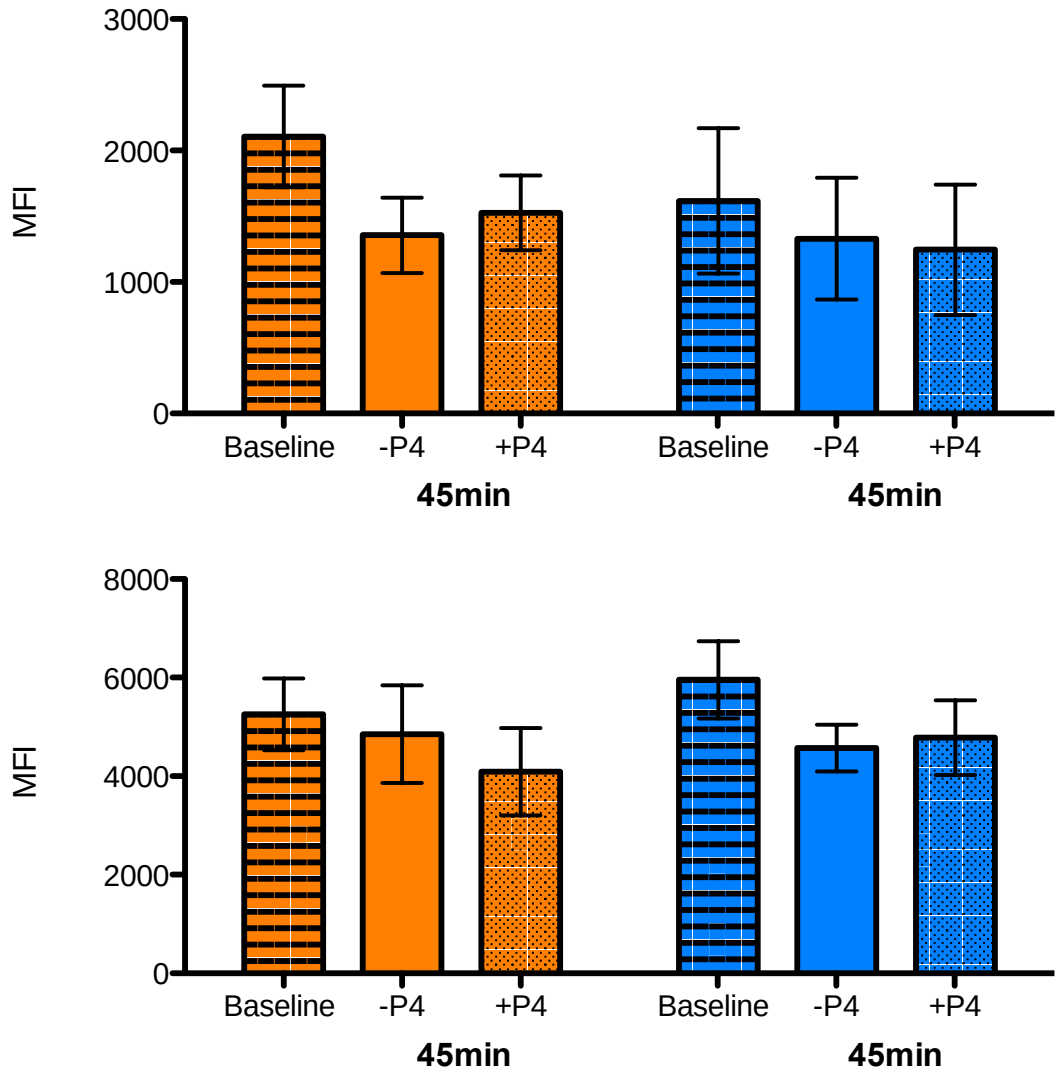


Figure 69 Effect of P4 on expression of CD64 (FcγRI) and CD16 (FcγRIII) on peripheral blood neutrophils
Peripheral blood neutrophils were isolated from CAP patients before incubating for 45 minutes in the presence of the P4 peptide. Cells were washed, stained with anti-CD64 and CD16 antibodies and expression levels determined by flow cytometry. Patients are divided into responders (orange, n=8) and non responders (blue, n=7). There was no significant difference in expression after treatment.

8. Complement receptor expression

No difference was observed in the expression of CD35 (CR1) or CD11b (CR3) between groups at baseline or after incubation with P4 peptide (Figure 70 and Figure 71).

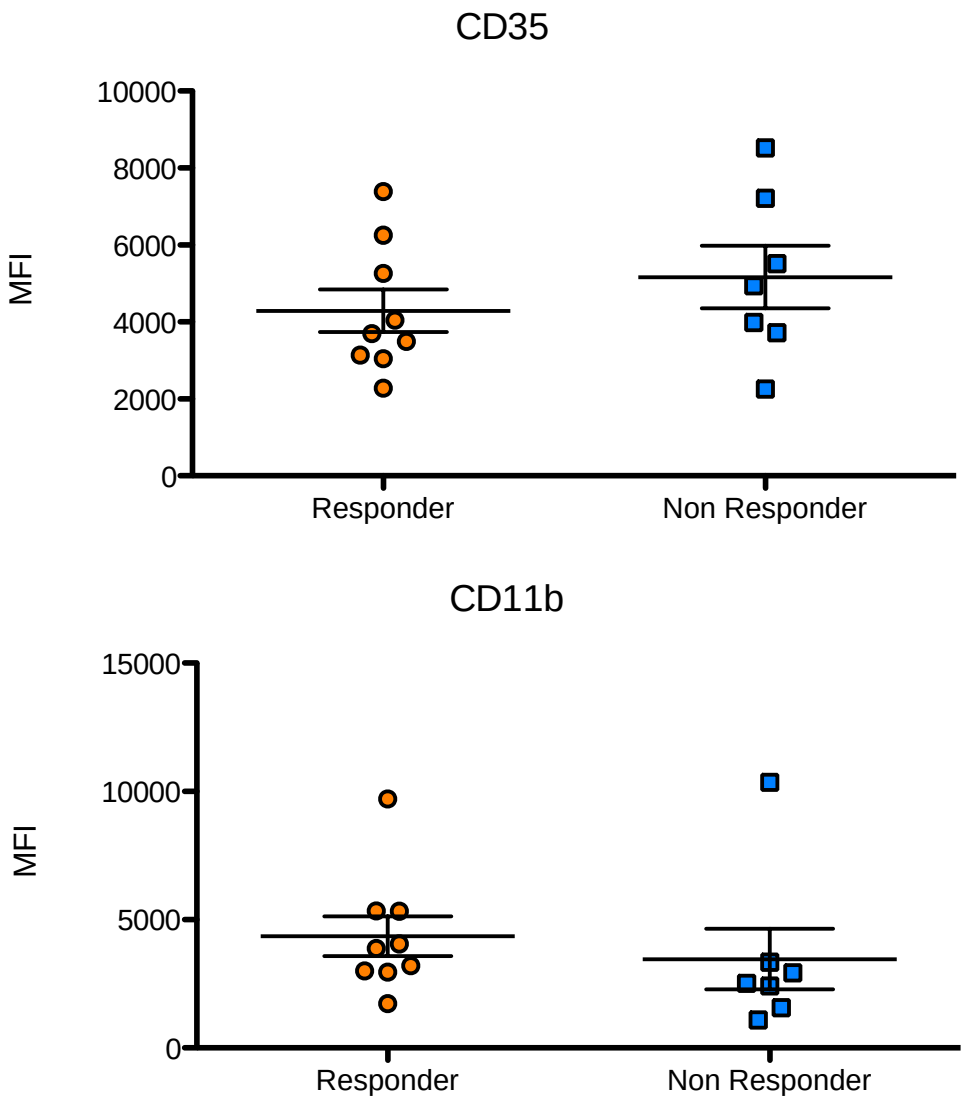


Figure 70 Complement receptor expression – CD35 and CD11b (CR1 and CR3) Complement receptor expression on neutrophils from patients (n=9 responders, n=7 non responders, n=7 patients lacked sufficient neutrophil numbers for testing or the sample was received too late for testing) using flow cytometry. There was no significant difference between the two groups in the expression level of CD35 p=0.4 or CD11b *p*=0.1 Mann Whitney test.

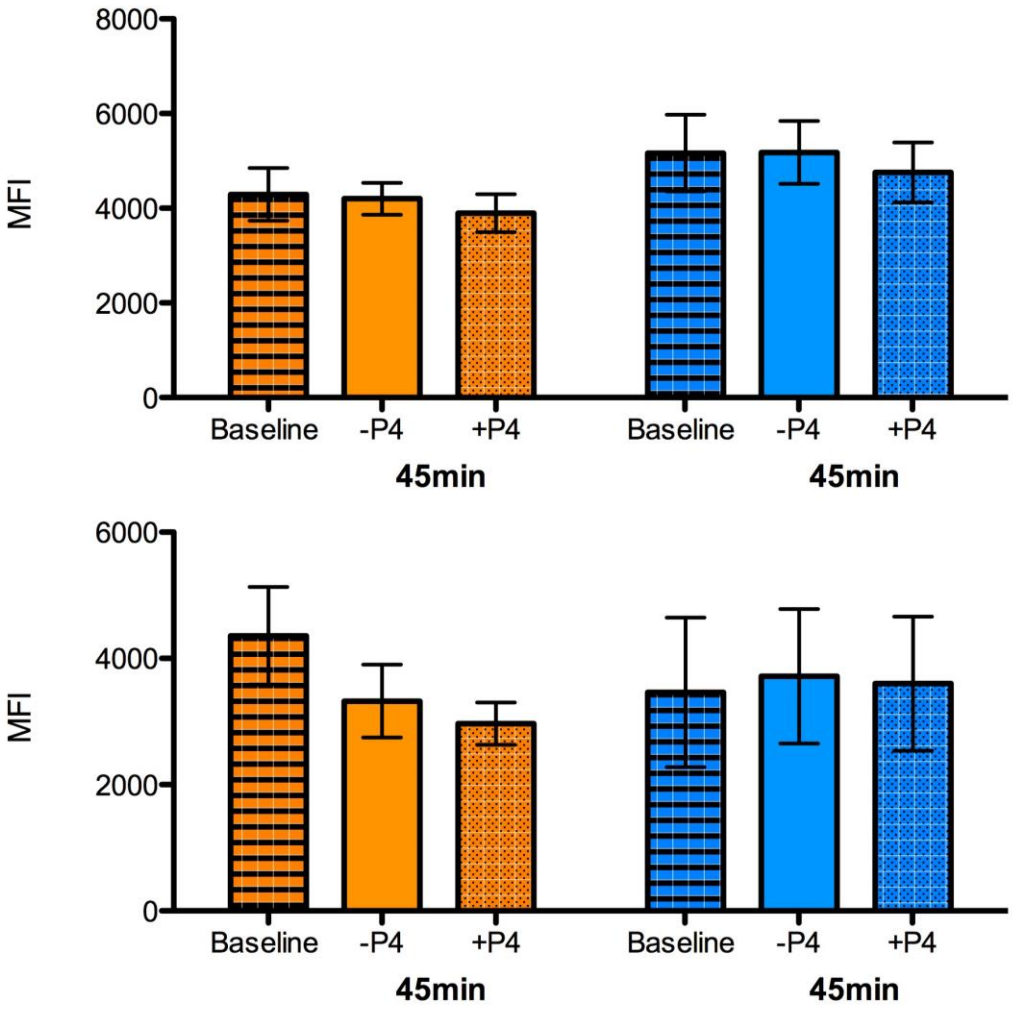


Figure 71 Effect of P4 on expression of CD35 and CD11b on peripheral blood neutrophils

Peripheral blood neutrophils were isolated from CAP patients before incubating for 45 minutes in the presence of the P4 peptide. Cells were washed, stained with anti-CD35 (CR1) and CD11b (CR3) antibodies and expression levels determined by flow cytometry. Patients are divided into responders (orange, n=8) and non responders (blue, n=7). There was no significant difference in expression after treatment.

CD66b is a marker of neutrophil activation, Figure 72 shows no difference was observed in the expression of CD66b between groups at baseline or after stimulation with P4 peptide as shown in Figure 73. Although it is of note that the non responder with the highest MFI (over 30,000) was the patient that had reduced killing in response to the P4 peptide which could suggest that there is of neutrophil activation after which P4 may have a negative effect on killing.

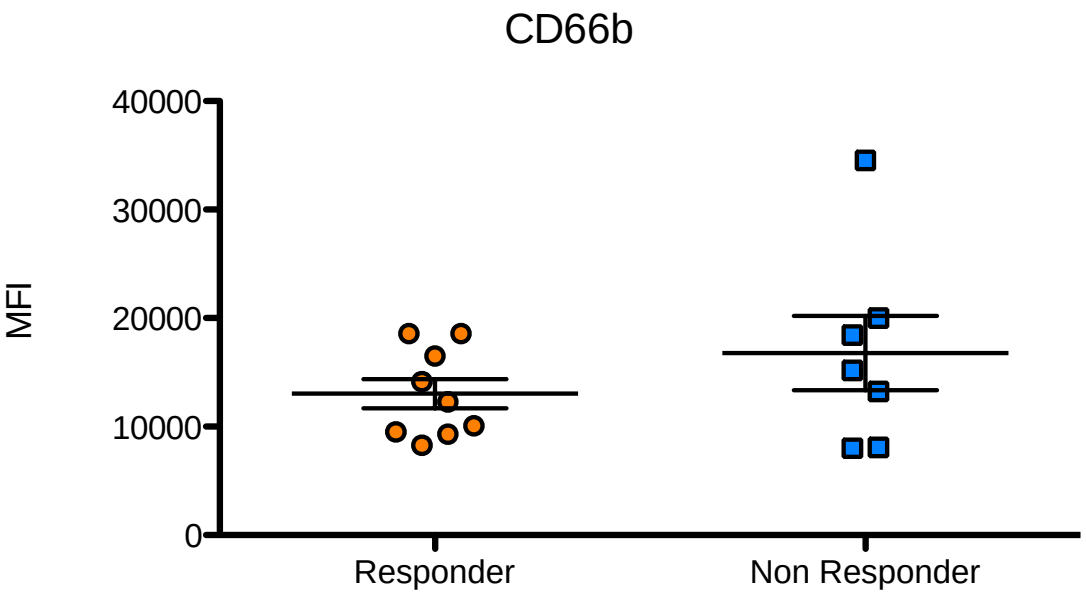


Figure 72 CD66b (CEACAM8) expression
CD66b (CEACAM8) expression on neutrophils from patients (n=9 responders, n=7 non responders, n=7 patients lacked sufficient neutrophil numbers for testing or the sample was received too late for testing) using flow cytometry. There was no significant difference between the two groups in the expression level *p*=0.6 Mann Whitney test.

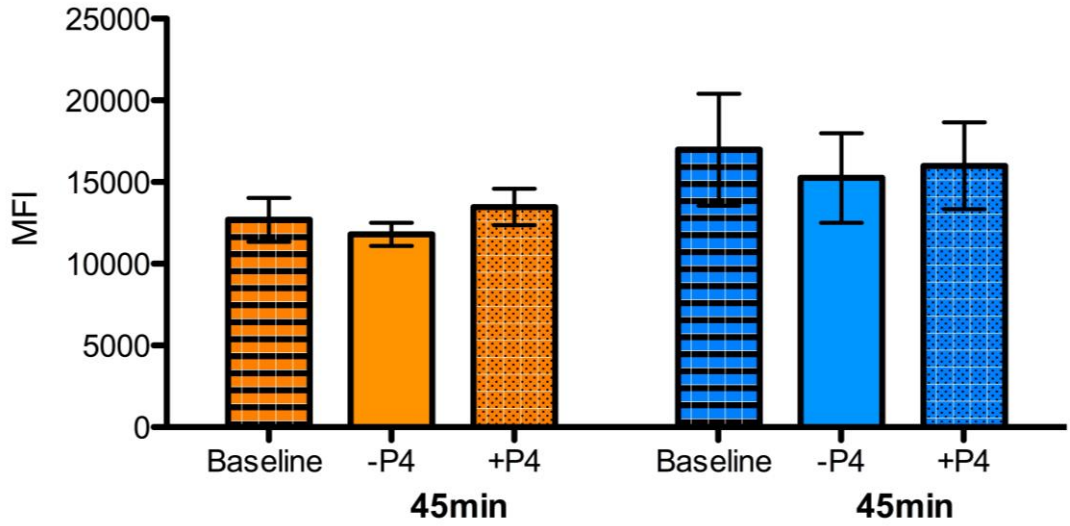


Figure 73 Effect of P4 on expression of CD66b on peripheral blood neutrophils
Peripheral blood neutrophils were isolated from CAP patients before incubating for 45 minutes in the presence of the P4 peptide. Cells were washed, stained with anti-CD66b antibodies and expression levels determined by flow cytometry. Patients are divided into responders (orange, n=8) and non responders (blue, n=7). There was no significant difference in expression after treatment.

9. IL-8R expression

Expression of the IL-8R on neutrophils isolated from the CAP patients was measured at baseline and after 45 minutes of stimulation with the P4 peptide. No difference was observed between responders and non responders in their baseline levels of IL-8R expression (Figure 77).

After 45 minutes of treatment of with P4 peptide both groups saw a significant decline in the expression of the IL-8R on the cell surface. Responders saw a drop of 53% whilst non responders saw a drop of 33% ($p<0.001$ and $p<0.01$ respectively) (Figure 74).

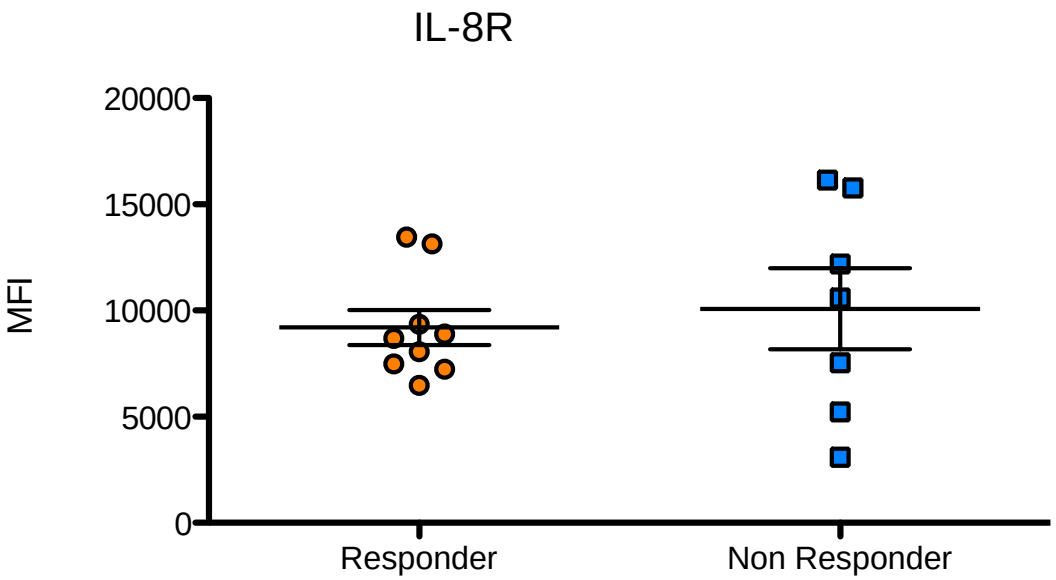


Figure 74 IL-8 receptor (CXCR1) expression
IL-8 receptor (CXCR1) expression on neutrophils from patients (n=9 responders, n=7 non responders, n=7 patients lacked sufficient neutrophil numbers for testing or the sample was received too late for testing) using flow cytometry. There was no significant difference between the two groups in the expression level. Mann Whitney test *p*=0.7.

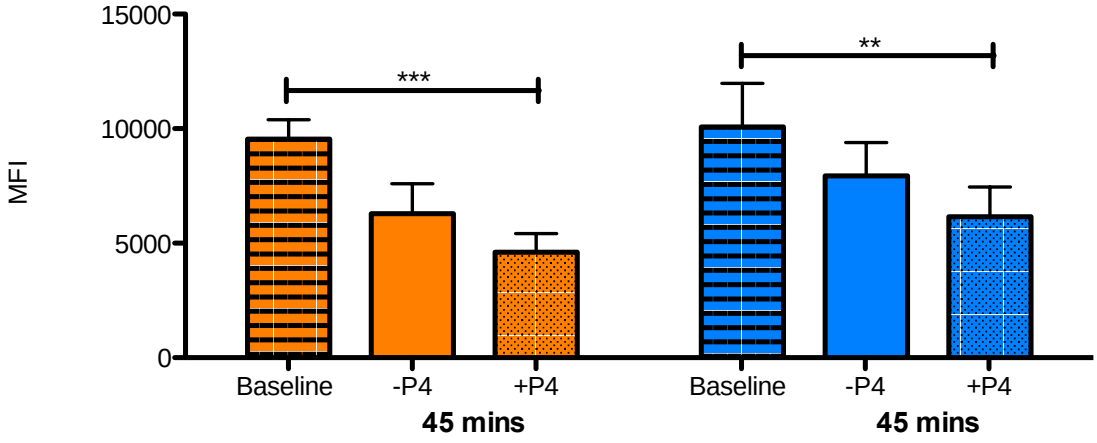


Figure 75 Effect of P4 peptide on IL-8R expression on peripheral blood neutrophils
Peripheral blood neutrophils were isolated from CAP patients before incubating for 45 minutes in the presence of the P4 peptide. Cells were washed, stained with anti-IL-8Rα (CD181/CXCR1) antibodies and expression levels determined by flow cytometry. Patients are divided into responders (orange, n=8) and non responders (blue, n=7). Both the responders and non responders showed a statistically significant drop from their baseline IL-8R expression after P4 treatment (responders p <0.0001, non responders p<0.001, Friedman test, ***= p<0.001, **= p<0.01 Dunn's Multiple Comparison test).

10. CD88 Expression

Expression of the CD88 on neutrophils isolated from the CAP patients was measured at baseline and after 45 minutes of stimulation with the P4 peptide. No difference was observed between responders and non responders in their baseline levels of CD88 expression (Figure 76). After 45 minutes of treatment of with P4 peptide both groups saw a significant decline in the expression of the CD88 on the cell surface. Responders saw a drop of 36% whilst non responders saw a drop of 10% ($p<0.01$ and $p<0.05$ respectively) (Figure 77).

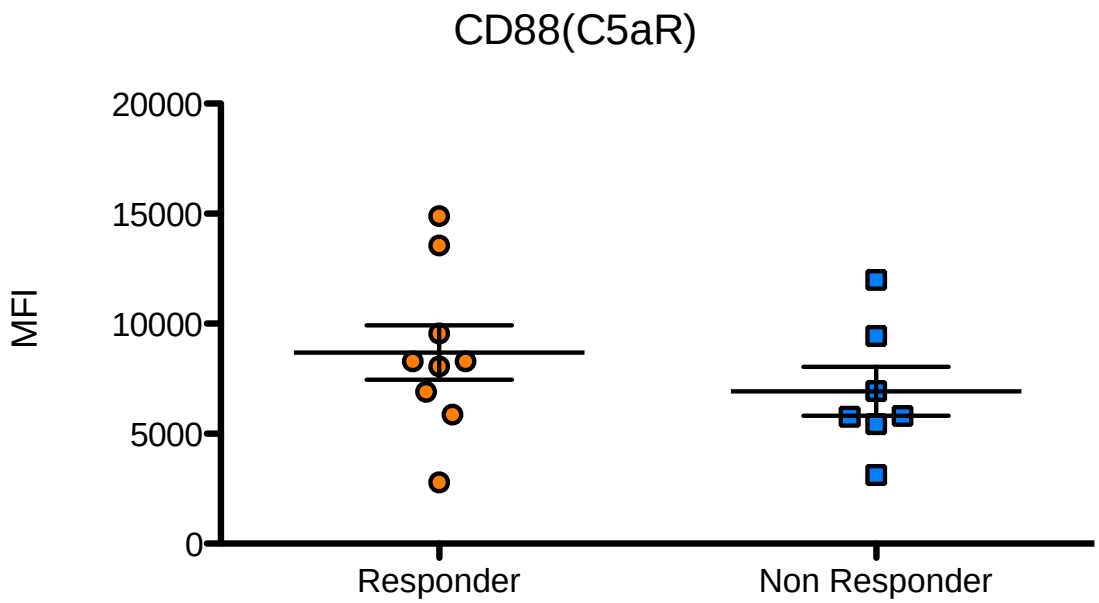


Figure 76 CD88 expression
CD88 expression neutrophils from patients (n=9 responders, n=7 non responders, n=7 patients lacked sufficient neutrophil numbers for testing or the sample was received too late for testing) using flow cytometry. There was no significant difference between the two groups in the expression level.

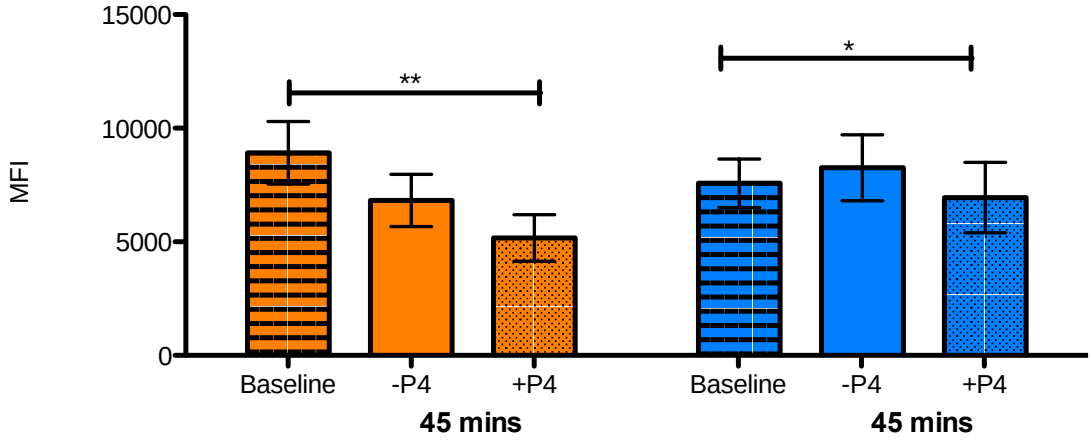


Figure 77 Effect of P4 peptide on CD88 expression on peripheral blood neutrophils
Peripheral blood neutrophils were isolated from CAP patients before incubating for 45 minutes in the presence of the P4 peptide. Cells were washed, stained with anti-CD88 antibodies and expression levels determined by flow cytometry. Patients are divided into responders (orange, n=8) and non responders (blue, n=7). Both the responders and non responders showed a statistically significant drop from their baseline CD88 expression after P4 treatment (responders p <0.00, non responders p<0.01, Friedman test, **= p<0.01, *= p<0.05 Dunn's Multiple Comparison test). The responders saw a mean drop from baseline of 36% whilst the non responders saw a drop of 10%. Untreated neutrophils showed a drop from baseline of 20% and an increase of 9% for responders and non responders respectively.

11. Alveolar Macrophages

Bronchoalveolar lavage (BAL) samples were collected from 3/23 patients in the study. Low sample numbers were due to the patients only receiving a research lavage if they needed one clinically. This meant lavages were often missed due to them being performed at short notice or overnight.

Of the three BAL samples collected OPKs were performed successfully on two. The first sample received the bacterial dose (500 CFUs per well) was too low to show a difference between groups as the macrophages were very active, subsequent assays were performed with a ten fold higher dose.

Figure 78 shows the results of these two OPKs. One patient showed an increase in killing index with P4 treatment from 72% to 93%. This patient was designated the letter S in the neutrophil studies and was a non responder. The other patient, who was designated G in the neutrophils and was a responder failed to show an improvement in killing in the alveolar macrophage OPK with a killing index of 86% at baseline and 89% with P4 treatment.

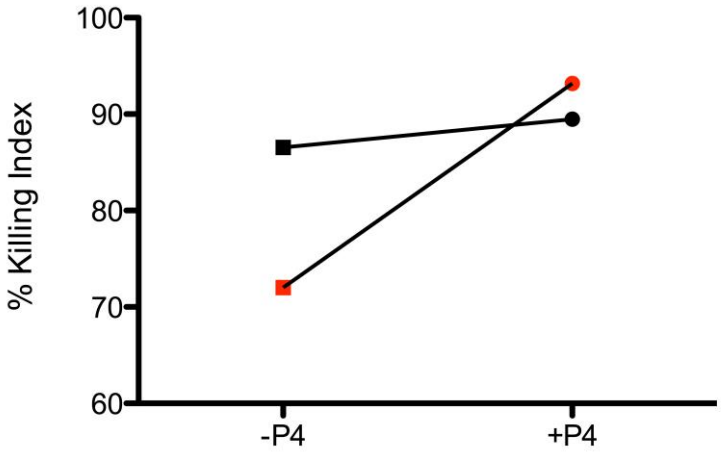


Figure 78 Alveolar macrophage OPK from CAP patients
OPK using alveolar macrophages isolated from patient BAL samples. Patient S is indicated in red and patient G in black.

D. Summary of results

Neutrophils and alveolar macrophages from healthy controls showed increased phagocytosis after treatment with P4 *ex vivo* in line with what as been demonstrated in previous studies (162).

Treatment of patient peripheral blood neutrophils with P4 peptide increases pneumococcal killing in 60% of the severe community-acquired pneumonia patients recruited to our study (p=0.002). A differential effect was observed with some patient neutrophils (40%) not showing a response to P4 peptide treatment in the OPK assay. However, the study was not sufficiently powered enough to fully decipher the reasons for these differences but there was a trend towards both a higher level of infection severity in non-responders (SOFA score p=0.0687) and a higher baseline level of opsonophagocytosis (p=0.0832) reducing the augmentative effect of P4.

There was a weak yet significant correlation between total blood neutrophil count and the difference in pneumococcal killing with P4 treatment (R squared value = 0.3, p=0.0019). Patients' serum cytokine data also showed a significantly higher level of both IL-8 and IL-10 in the serum of non responders (p=0·03 and 0·01 respectively) indicative of both neutrophil recruitment and anti-inflammatory effects.

Cell surface marker data showed no significant difference in the baseline levels of any of the surface receptors measured. When cell surface markers were measured after treatment with P4 there was a statistically significant decrease from baseline levels in CD88 and IL-8R in both P4 peptide responder and non responder groups. CD32 also dropped in expression level

with P4 treatment but only in the responder group, non responders maintained baseline values.

E. Discussion

This is the first study to look at the *ex vivo* effects of the P4 peptide on human peripheral blood neutrophils during infection. The OPK assay was used as our end point for assessing efficacy of the peptide in our patient cohort.

1. OPK

A significant increase in pneumococcal killing index was seen for 60% of the patients studied. This is an encouraging result suggesting that P4 treatment could potentially be of benefit to patients suffering from severe CAP and sepsis.

An important factor that must be taken into consideration however is that the OPK assay, being an *ex vivo* assay, may not give the whole picture as to which patients may or may not benefit from P4 treatment. The OPK assay doesn't give a realistic representation of the conditions with the patients as the neutrophil numbers are adjusted to a standard number and the various cytokines, chemokines and other plasma components which could influence neutrophil function are removed. A whole blood phagocytosis assay may reveal differences between patients which aren't seen in the neutrophil OKP used in this study. Another shortfall of this assay is that the protocol used for isolation of neutrophil precludes the isolation of the most immature neutrophils as their low density results in them being deposited in the PBMC fraction after density centrifugation (201). These immature neutrophils have decreased function compared with mature neutrophils and are thought to be

more pro-inflammatory, hence would be an important population to study (202).

2. Receptor expression

a) CD88 and IL-8R

This study looked at cell surface markers on neutrophils isolated from CAP patients and showed decreases in cell surface expression for both CD88 and IL-8R: two receptors important to neutrophil activation and chemotaxis, these changes occurred in both the responder and non responder group (203-205). Due to the short period of P4 stimulation (45 mins) that induced these reductions in receptor expression the most likely explanations are that the receptors were either internalised or cleaved from the cell surface. This could be confirmed by measuring levels of the receptor in the cell culture supernatant or with confocal imaging with fluorescently labelled receptors to capture receptor internalisation. Release of neutrophil elastase (NE) is mediated in part by the binding of C5a to CD88 and results in the cleavage of CD88 from other cells (127). It would be interesting to see if NE had been released into the supernatant in the presence of P4 and whether or not this is affected by the presence of FBS (as a potential source of C5a).

This augmentation of receptor expression helps support the idea of the peptide having effects beyond just enhancing phagocytosis, such as augmenting timing and number of neutrophil infiltrating infection sites with P4 treatment as shown in mouse models of pneumococcal infection treated with P4 (167).

b) FcγR expression

An interesting finding from this study was the effect of P4 on the expression of CD32 (FcγRII) on patient neutrophils, as one would not expect a decline in CD32 to result in increased phagocytosis . CD32B, one of the two isotypes of the CD32 molecules that has an inhibitory function, but it is not normally thought to be expressed by neutrophils though it can be induced under certain conditions (206). The ratio of CD32A:CD32B mRNA has been shown to be important in setting the threshold of activation in neutrophils (207). The antibodies used to detect CD32 in our study cannot distinguish between CD32A and CD32B so it is not possible to say if only one or both of these receptors is being affected by P4 treatment but is definitely a line of research worth pursuing in future studies.

Expression levels of CD64 (FcγRI) and CD16 (FcγRIII) were not significantly different between responders and non responders, nor were any changes in expression observed after stimulation with P4 peptide. As CD64 has very low levels of expression in resting neutrophils it not be possible of P4 to induce further expression in CAP patients as it is already optimally expressed (177). Differences in the level of CD16 between patients may not have been evident because of the inability of the neutrophil separation method used to isolate the immature neutrophils which would have had lower levels od CD16 expression (202).

3. Inflammatory Markers

Another finding, which could be significant when selecting patients who would benefit from P4 therapy, is the relationship between serum IL-8 and IL-

10 levels and response to treatment. Patients with high levels of IL-8 and IL-10 were more likely to be non responders. This could be due to IL-8 priming neutrophils for phagocytosis and would in turn explain the higher baseline level of phagocytosis in non responders (208). Alternatively IL-10 has been shown to induce a refractory period in neutrophils during which activation in response to bacterial stimuli is blocked after an initial exposure to the stimuli in the presence of IL-10, which could explain why the non responders failed to show a improvement in phagocytosis with P4 peptide treatment (209). The interplay between IL-8, IL-10 and P4 treatment is worth investigating further to examine whether these cytokines are having an effect on the ability of P4 to increase phagocytic activity.

4. Effect of time

If P4 therapy went through to clinical trials, its use is envisage as it being administered in a similar time frame to initial antibiotic treatment in sepsis i.e. within the first hours of sepsis being suspected. In this study samples were received from 13 to 110 hours after admission. No effect of time was observed in either the patient response to the drug or in severity of disease in patients.

5. Alveolar macrophages

The results of the alveolar macrophage OPK have too small a sample size to draw any definitive conclusions as to whether P4 can improve phagocytosis by macrophages in the lungs of CAP patients but they do highlight that it is possible that patients who don’t show a response to P4 in their blood neutrophils could still benefit from P4 administration directly into the lungs.

6. Summary

In summary this study has shown that treatment with P4 may have benefits for patients with severe CAP but as improvements in phagocytosis were not seen in all patients it would be important to investigate further what factors determine whether or not the neutrophils respond to treatment; patient plasma IL-8 and IL-10 levels may be worth investigating further which patients would benefit from P4. This study also showed that treatment with P4 did alter expression of CD32, CD88 and the IL-8R – it would be essential to investigate further what the effect of alter the cell surface expression of these receptors has during infection.

Chapter V. Mechanism of P4 action

A. Introduction

The working theory on the mechanism of P4 action is that it increases the levels of FcγR expression on phagocytic cells, which in turn leads to an increase in phagocytosis (167). This chapter looks at the effect of P4 on naïve mice *in vivo* and *ex vivo*.

1. P4 treatment of naïve mice *in vivo*

Mice were treat with P4 for 24 hours before being culled and their neutrophils analysed by flow cytometry to look at the effects of P4 on expression of CD88 , CD35/21 (CR1/2) and IL-8R.

2. P4 treatment of naïve mouse neutrophils *ex vivo*

Neutrophils isolated from naïve mouse bone marrow were treated with P4 in OPKs. Expression of CD64 and CD32/16 was measured after P4 treatment.

B. Method and Materials

1. P4 treatment of naïve mice *in vivo*

Female CD-1 mice aged 6-8 weeks were treatment with P4 peptide and then culled after 24 hours and blood collected for flow cytometry analysis.

P4 peptide, dissolved in DEPC treated water, was administered via a 50 µl intravenous injection into the tail vein at a concentration final concentration per mouse of 1 µg, 10 µg and 100 µg. Control animals received an injection of equivalent volume of sterile PBS.

2. P4 treatment of naïve mice *ex vivo*

a) Tissue collection and neutrophil isolation

Naïve mice were culled by cervical dislocation after which the femurs and tibias were collected and washed in 70% ethanol. The epiphyses were removed and the bone marrow flushed out of with a needle and syringe containing RPMI 1640 supplemented with 2 mM EDTA and 10% FBS.

Cells were pelleted by centrifuging at 400 g, the supernatant removed and red blood cells lysed in 0.2% saline. Cells were pelleted again by centrifuging at 400 g, the supernatant removed and cells resuspended in 2 ml of RPMI 1640 supplemented with 2% EDTA and 10% FBS.

Histopaque 1119 and histopaque 1077 were layered over one another (histopaque 1077 on top), 2 ml of each, in a 15 ml centrifuge tube. The cell suspension was then layered on top of the histopaque and the tubes centrifuged at 800 g for 30 mins with no brake. The PBMC fraction was

discarded and the neutrophil fraction collected. Neutrophils were counted with a haemocytometer and viability assessed via trypan blue exclusion.

b) OPK and P4 treatment

OPKs were performed as described in section IV.B.11. Neutrophils were resuspended in RPMI to give a concentration of 1.7 x 10^6 cells/ml. Neutrophils were seeded into 96 well plates, 180 µl per well. Wells were treated with either: P4 20 µl of 3 mg/ml dissolved in DEPC treated water or with DEPC treated water. Cells were incubated for 1 hour at 37 °C, 5% CO_2.

c) Receptor Expression

Cells for receptor expression analysis were treated with P4 and DEPC treated water. The antibodies detailed in Table 17 with corresponding isotype controls were used for flow cytometry analysis of mouse tissue and leukocytes. Antibodies were used at a dilution of 1:250. Staining procedure is detailed in section II.B.4.b. Sample acquisition was performed on the FACS Calibur (BD Biosciences, USA) and analysis was performed with FlowJo 8.7 (Tree Star, USA).

Table 17 Mouse flow cytometry antibodies

Biolegend Product Code	Description
101325	APC anti-mouse CD16/32
139303	PE anti-mouse CD64 (FcγRI)
123417	APC/Cy7 anti-mouse CD21/CD35 (CR2/CR1)
149307	PerCP/Cy5.5 anti-mouse CD182 (CXCR2)
108405	FITC anti-mouse Ly-6G/Ly-6C (Gr-1)

C. Results

1. P4 treatment of naïve mice *in vivo*

Previous studies have shown the significant increases in the expression of FcγRs in naïve mice treated with P4 but no studies have looked at other receptors (167). CD88, CD35/21 (CR1/2) and CD182 (CXCR2) were chosen because of the evidence that complement may be an important factor in P4 function; OPK assays without complement present fail to show an improvement in phagocytosis with P4 treatment (162). CD182 (CXCR2) was chosen because of the differences observed in the human *ex vivo* CAP study in IL-8R expression with P4 treatment.

Figure 79 shows the expression of CD88 on neutrophils form mice treated *in vivo* for 24 hours with P4 peptide. The figure shows a dose dependent increase in CD88 expression with the highest expression found with 100 µg of P4 per mouse. The difference in expression was significantly different for control vs. 100 µg of P4 (p=0.005).

Figure 80 shows the expression of CD35/21 on neutrophils form mice treated *in vivo* for 24 hours with P4 peptide. Levels of CD35/21 expression with P4 treatment saw a 2 fold increase at a P4 concentration of 1 µg and a modest increase with 100 µg, these values were approaching significance (p=0.05).

Figure 81 shows significantly higher expression of CD182 with 1 µg treatment with P4. Increases were not observed with 10 or 100 µg treatments.

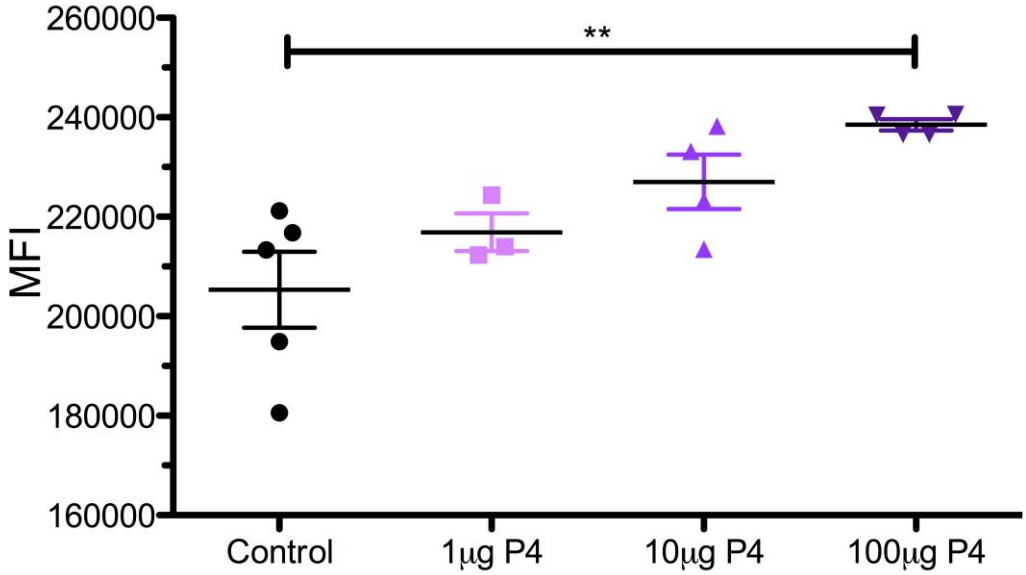


Figure 79 Effect of P4 on expression of CD88 on neutrophils of naïve mice
Mice n=3-5 per group with injected intravenously with 50 µl of either P4 or PBS. Mice were culled 24 hours after treatment and blood was collected, red blood cells were lysed and cells stained with anti-Gr-1, CD11b and CD88 antibodies for subsequent analysis by flow cytometry. Neutrophils were identified as GR-1+, CD11b+ cells. Plotted as mean and SEM, analysed by one-way ANOVA p=0.0074, $^{**}p<0.005$

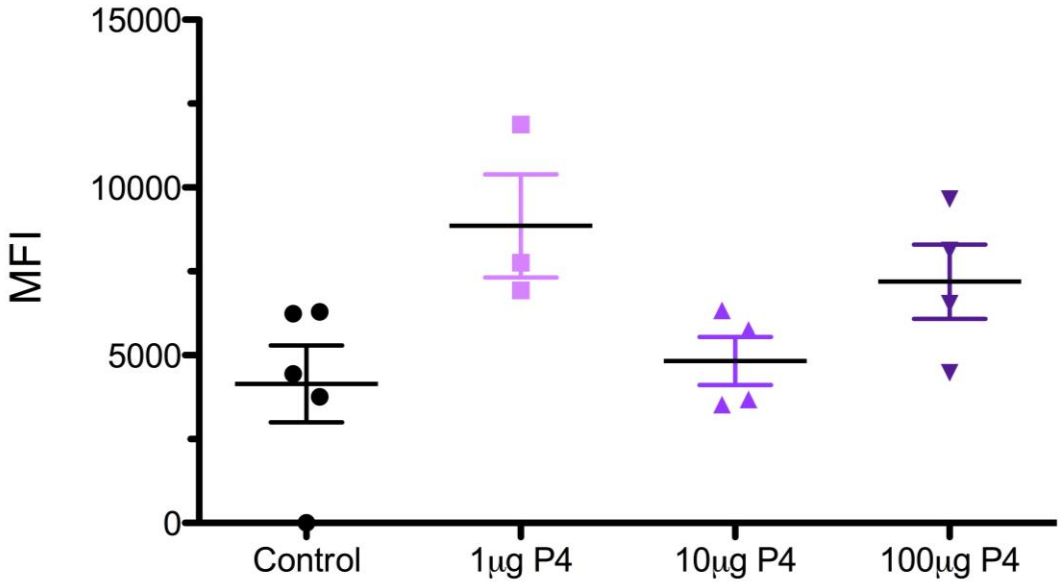


Figure 80 Effect of P4 on expression of CD35/21 (CR1/2) on neutrophils of naïve mice
Mice n=3-5 per group with injected intravenously with 50 µl of either P4 or PBS. Mice were culled 24 hours after treatment and blood was collected, red blood cells were lysed and cells stained with anti-Gr-1, CD11b and CD35/21 antibodies for subsequent analysis by flow cytometry. Neutrophils were identified as GR-1+, CD11b+ cells. Plotted as mean and SEM, analysed by one-way ANOVA p=0.05

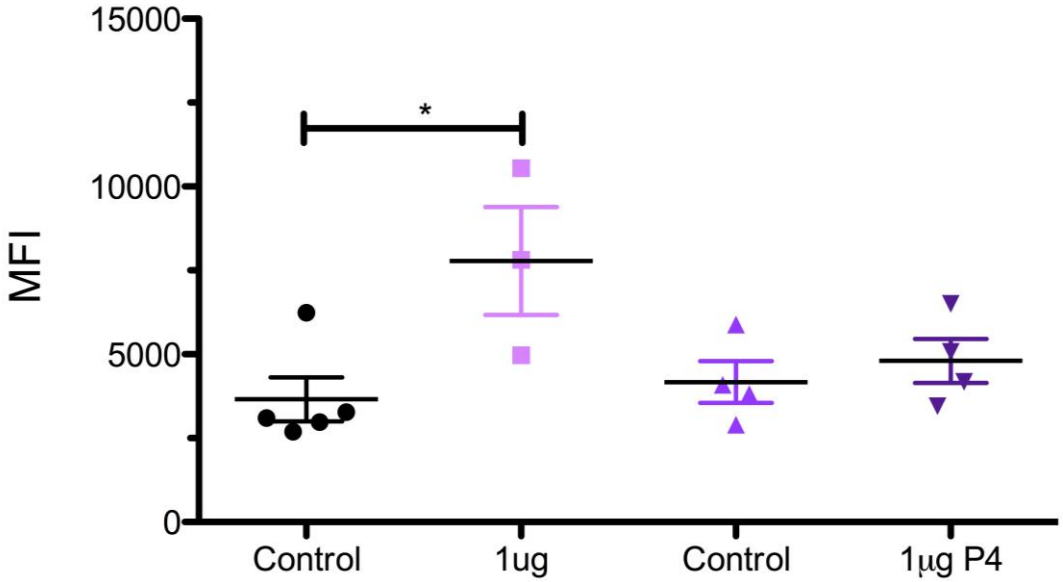


Figure 81 Effect of P4 on expression of CD182 (CXCR2) on neutrophils of naïve mice

Mice n=3-5 per group with injected intravenously with 50 µl of either P4 or PBS. Mice were culled 24 hours after treatment and blood was collected, red blood cells were lysed and cells stained with anti-Gr-1, CD11b and CD182 antibodies for subsequent analysis by flow cytometry. Neutrophils were identified as GR-1+, CD11b+ cells. Plotted as mean and SEM, analysed by one-way ANOVA p=0.03, *p<0.05

2. P4 treatment of bone marrow neutrophils form naïve mice *ex vivo*

As neutrophils are released in high numbers from the bone marrow during infection it was important to establish whether these neutrophils respond to P4 treatment. Figure 82 shows the difference in killing index with P4 treat of neutrophils isolated from the bone marrow of naïve mice. The neutrophils showed a significant increase in killing with P4 treatment (p=0.007). It was not possible to compare killing between blood and bone marrow neutrophils because the yields from neutrophils isolation from mouse blood is too low to per an OPK assay with.

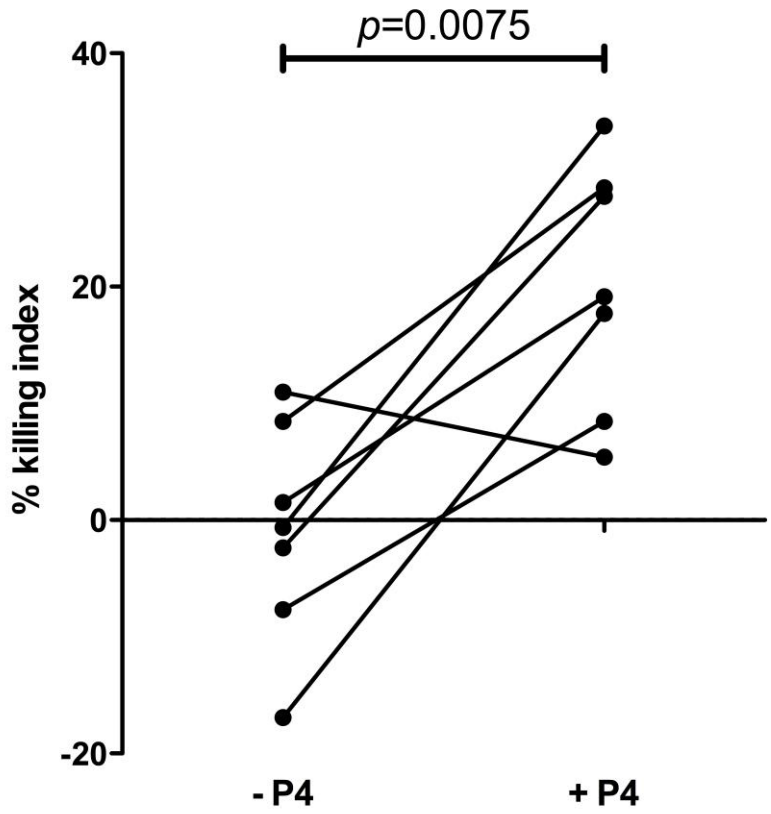


Figure 82 Difference in killing index with P4 treatment - naïve bone marrow neutrophils
Killing index of naïve bone marrow neutrophils in OPK assay with and without P4 treatment. The neutrophils from the bone marrow of 7 mice were isolated and individual OPKs performed for each mouse. Analysed by paired t-test p=0.0075.

Figure 83 and Figure 84 show the effect of P4 treatment on expression of CD64 (FcγRI) and Cd32/16 (FcγRII/III) on neutrophils isolated from the bone marrow of naïve mice. Significant increases in CD64 were seen with P4 treatment (p=0.03), a 2 fold increase in expression was observed. Increases in the expression of CD32/16 were only seen in 3/6 mice with P4 treatment (p=0.4). Why the neutrophils from some mice appeared to have a response to P4 with regards to CD32/16 expression whilst others did not is unclear.

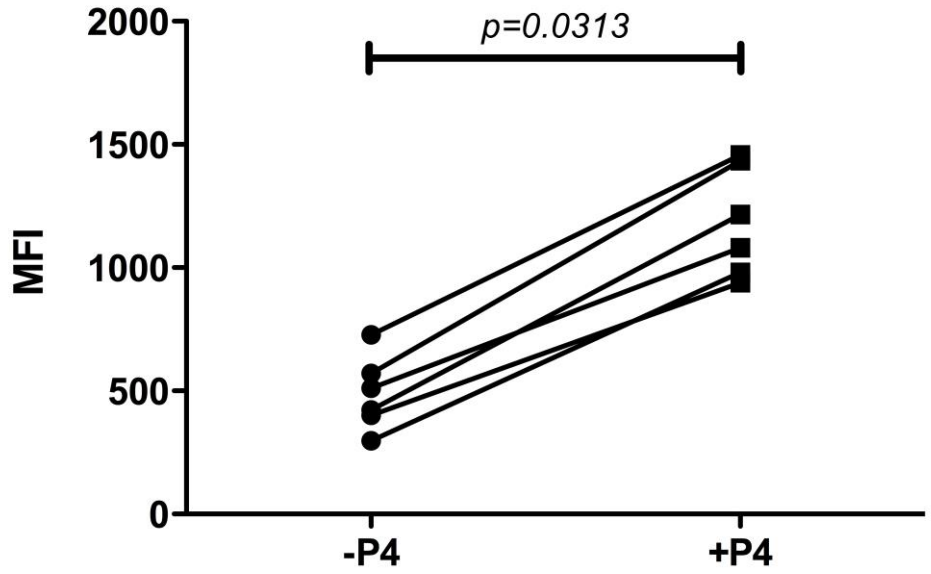


Figure 83 Effect of P4 treatment on expression of CD64 (FcγRI) on naïve bone marrow neutrophils
The bone marrow from 6 mice was collected and neutrophils isolated, neutrophils were treated with P4 or DEPC treated water for 1 hour before cells were stained with CD64 antibodies for subsequent analysis by flow cytometry. Analysed by paired t-test *p*=0.03.

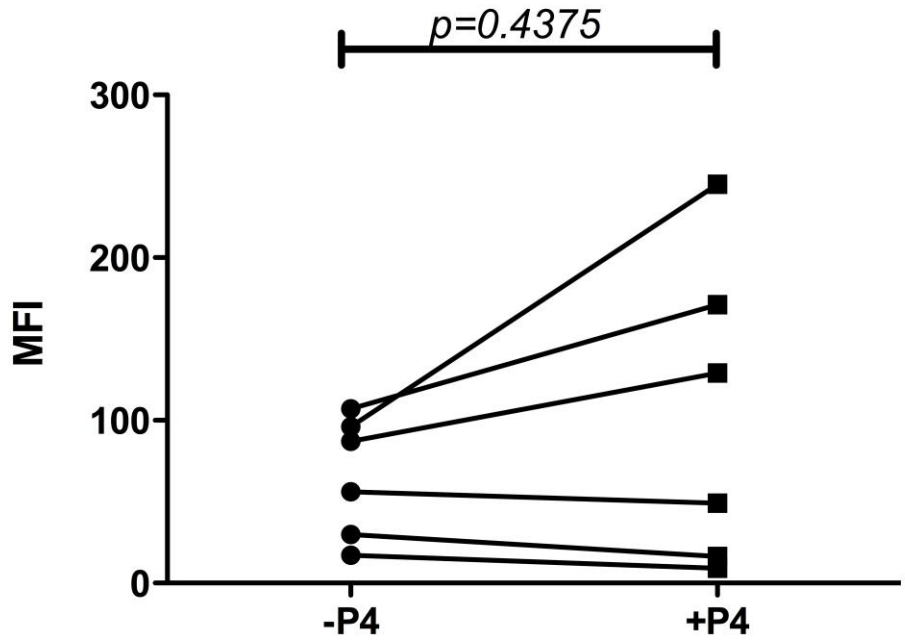


Figure 84 Effect of P4 treatment on expression of CD32/16 (FcγRII/III) on naïve bone marrow neutrophils
The bone marrow from 6 mice was collected and neutrophils isolated, neutrophils were treated with P4 or DEPC treated water for 1 hour before cells were stained with CD32/16 antibodies for subsequent analysis by flow cytometry. Analysed by paired t-test p=0.4

D. Results summary

1. P4 treatment of naïve mice *in vivo*

Treat of naïve mice *in vivo* with P4 resulted in significant increases expression of CD88 in a dose dependent manner. Increases in CD182 (CXCR2) and CD35/21 (CR1/2) were also observed but unlike CD88 the highest expression was observed with 1 µg treatment and no dose dependent relationship was seen.

2. P4 treatment of bone marrow neutrophils form naïve mice *ex vivo*

OPKs with bone marrow neutrophils from naïve mice resulted in significant increases in killing index with P4 treatment.

Ex vivo treatment with P4 of bone marrow neutrophils from naïve mice resulted in significant increases in CD64 after 1 hour. Differences in CD32/16 expression were observed in some mice but not others and overall was not significant.

E. Discussion

1. P4 treatment of naïve mice *in vivo*

Increased expression of receptors in naïve mice, other the CD32/16, has not previously been shown (167). The ability of P4 to modulate the expression of other receptors is an important finding with regards the mechanism of P4 function. CD88 showed significant increases in expression after treatment with P4 and the fact that these changes in expression in detectable 24 hours after treatment with P4 peptide suggests that the effects of P4 on cells could be long lasting. It would be interesting to see how long the effects of P4 last in cells and how they change over time as this could effect the dosing schedules used in treatment. C5a is an extremely potent neutrophil chemoattractant so increased expression of the receptor could explain increases in neutrophils seen during infection with P4 treatment. Increases in CD182 could also contribute to increases in neutrophils. Binding of C5a to the CD88 has also been shown to play a role in regulation of CD16 expression, so chances is CD16 expression could also be a result of changes in the expression CD88 (115).

Significant increases in CD182 expression were also observed after P4 treat, however they did not display a dose dependent effect with the highest expression being in the group treated with 1 µg of P4. CD32/16 expression showed increases close to significance (p=0.0515) with P4 treatment but like the results seen for CD182 expression, levels were highest with 1 µg of P4. With this in mind, further studies should be performed looking at the dose dependent effects of P4 on receptor expression.

2. P4 treatment of bone marrow neutrophils form naïve mice *ex vivo*

The ability of neutrophils isolated from the bone marrow of naïve mice to respond to P4 in the OPK, which resulted in a significant increase in killing index with P4 treatment, is important as during infection large numbers of immature neutrophils are released from the bone marrow and in sepsis have been show to have reduced function compared with mature neutrophils normally found in circulation (202). This result should be followed up with studies of the effect of P4 peptide on neutrophil function during infection.

The neutrophils showed significant increases in expression of CD64 with P4 treatment. Baseline levels were very low in untreated cells, which helps support the theory that CD64 is not increased with P4 treatment during infection because it is already optimally expressed.

Expression of CD32/16 was not significantly increased in all mice treated with P4, the mice with the lowest levels of CD32/16 without treatment with P4 failed to show increases in expression whilst those with higher expression levels did show an increase. As an increase in expression this rapid is likely to be due to transport of intracellular stores of receptors to the cell surface it would be interesting to see if this mice had differed in the levels of stored receptors with the cell and hence were not capable of increasing their surface expression.

Chapter VI. Discussion and Future work

A. Discussion

1. Animal models

The two animal models utilised in this study yielded very different results, which are briefly summarised in Table 18. The *E. coli* infection model was far more severe than the *K. pneumoniae* infection model, with control animals progressing to their humane endpoint within eight to ten hours of infection whilst controls in the *K. pneumoniae* model started showing signs of severe infection from 36 hours onwards and the mean survival time for controls was 78 hours.

The inflammatory response observed in the *E.coli* model was also markedly higher than in the *K. pneumoniae* model with CXCL1 levels in plasma almost 300 fold higher and C5a levels 100 fold higher in *E. coli* infected mice. This excessive inflammatory response probably led to the very rapid decline in host survival in the *E. coli* infection model and therefore to see a protective effect of P4 treatment on mouse survival time would have needed some form of physiological support (such as fluid resuscitation, vasopressors and inotropic drugs), which is difficult to implement in animal models. Given the very rapid progression of infection it is likely that the high levels of inflammation was driven by high levels of LPS in *E. coli* infected mice. The infectious dose in the *E. coli* model was 100 fold higher than in the *K. pneumoniae* so it is possible that the initial inoculum contained substantial amounts of free LPS. In pilot experiments to set the dose for the *E. coli* model mice cleared the infection with doses of 10^6 CFUs/mouse and less. In future studies it would be interesting to establish whether there is a high

concentration of LPS in the dose and if removal of this could improve survival in the model.

The relatively slow progression of the *K. pneumoniae* infection allowed a larger window within which treatment could be administered i.e. treatment could be given before there was widespread inflammation in the model which could possibly counter the effects of P4 treatment.

Another difference between the two models which may explain disparities in the results in the relation to Tazocin + IVIG treatment is the site of infection in relation to site of administration of IVIG: in the *E. coli* model the infectious dose and the IVIG were both given via intraperitoneal injection whilst in the *K. pneumoniae* model the infectious dose was given via the intranasal route and the IVIG via intraperitoneal injection. With the IVIG being administered directly into the initial site of infection in *E. coli,* improvements were seen in survival time of Tazocin + IVIG treated mice when compared to treatment with Tazocin alone. This could be because by administering the IVIG in close proximity to the infection site it was better able to slow the dissemination of infection from the peritoneum into the blood. These differences in survival were not observed between Tazocin and Tazocin + IVIG treated mice in the *K. pneumoniae* model where IVIG would have had to cross into the blood before circulating into the lungs. Which leads to the question as to whether Tazocin + IVIG treatment in *E. coli* infected mice would have still given a survival advantage over Tazocin alone if it had been administered through an alternative route such as intravenously, as it would be in humans. It was not possible to test this theory in the mice, as intravenous injection of IVIG (as in human studies) is not well tolerated by the mice.

The *K. pneumoniae* infection model showed good efficacy of P4 treatment with P4-IVIG + Tazocin treatment resulting in 70% survival compared with 20% survival in Tazocin treated groups and 30% survival in Tazocin + IVIG treated groups. This increase in survival was likely due to the lower numbers of CFUs in mice treated with P4 compared to control. Increases in CD32/16 (FcγRII/III) expression in mice treated with P4 supports what has previously been published on the effects of P4 treatment on neutrophils and are likely to have resulted in in the lower numbers of CFUs (167).

Whether P4 treatment provides a benefit in *E. coli* infection is not as clear cut; survival times and CFUs recovered from tissue were not significantly different for both P4-IVIG + Tazocin and Tazocin + IVIG treated mice. Cytokines and neutrophil receptor expression also did not differ significantly with P4-IVIG + Tazocin compared to mice treated with just Tazocin. To better establish whether P4 treatment is beneficial in *E. coli* infection, a less severe model, that more closely replicates the human progression to sepsis and allows longer survival times, such as a cecal ligation and puncture model could be used (210).

Table 18 Comparison of *E. coli* and *K. pneumoniae* in vivo models

	E. coli	*K. pneumoniae*
Infection Route	Intraperitoneal	Intranasal
Inoculum per mouse (CFUs)	10^7	10^5
Dosing schedule post infection	1 and 4 hours	24 and 30 hours
Mean survival time of PBS control	8 hours	78 hours
Survival percentage in P4-IVIG + Tazocin	55% at 24 hours	70% at 7 days
Time pointed cull	7 hours	36 hours
Plasma Cytokines – PBS control	Very High CXCL1 Very High C5a High IL-10	High CXCL1 High C5a No detectable IL-10
Plasma Cytokines – P4-IVIG + Tazocin	Very High CXCL1 Lower levels of C5a Intermediate IL-10	High CXCL1 High C5a No detectable IL-10
Neutrophil percentage	Elevated in Tazocin and P4-IVIG + Tazocin treated mice	Elevated P4-IVIG + Tazocin treated mice only
CD64 expression on neutrophils	No difference	No difference
CD32/16 expression on neutrophils	Elevated in Tazocin and P4-IVIG + Tazocin treated mice	Elevated in P4-IVIG + Tazocin only
CD88 expression on neutrophils	Elevated in Tazocin and P4-IVIG + Tazocin treated mice	No difference

2. *Ex vivo CAP study*

The *ex vivo* study on the effects of P4 on neutrophils from patients with severe CAP showed that treatment with P4 significantly increased phagocytosis, with 60% of the patients in the study showed an increase in killing. The patient sample size of this study limited further conclusions such as which patients would benefit from P4 treatment, although the difference in SOFA scores between responders and non responders suggested that P4 efficacy declines as infection severity increases and hence the timing of treatment with P4 will likely have a major influence on treatment success. This relationship between infection severity and P4 efficacy was also demonstrated in the *E. coli* infection models, where it is likely that the rapid progression of the infection masked any benefits of P4 treatment.

The group of patients that did not respond to P4 treatment in the OPK had significantly higher levels of IL-8 and IL-10 in their plasma than those that did show a significant response. Further work to establish if and how IL-8 and IL-10 affect the ability of P4 to augment neutrophil function may help to advance understanding of P4's mechanism of action. High levels of IL-8 may mean that the neutrophils are already optimally primed to respond to infection whereas high levels of IL-10 are known to attenuate neutrophil responses to stimuli such as LPS and this function may not be able to be recovered with P4 treatment (208, 209, 211).

The results from the alveolar macrophage OPKs highlight that phagocytes from different sites (e.g. the lung and blood) may respond differently to P4

treatment, so for some patients administration of P4 directly into the lungs may be beneficial.

Changes to the expression of CD88 in both the animal models and the CAP patients and changes to IL-8R expression in the CAP patients suggest that there is more to the P4 mechanism than just increasing phagocytosis from augmentation of FcγRs. The fact that these changes were observed in naïve mice treated with P4 and not just during infection supports the idea that P4 may be directly impacting on the expression of CD88 and IL-8Rs and that this is not just a by product of reduced infection severity. The implications of these changes in receptor expression on neutrophil responses to infection need further investigation.

3. Future Work

P4 has received funding through the MRC in the form of a Developmental Pathway Funding Scheme grant to further development of P4 as a therapeutic. As part of this grant P4 is currently undergoing toxicity testing (results due September 2016), which will hopefully lead to phase I and II clinical trials. With the possibility of P4 being administered to human subjects in mind, further work should be performed to better understand the mechanism of action of P4. The rapid nature of the up-regulation of FcγRs by P4 in vitro supports the idea that the receptors are being released from intracellular stores but how this is triggered remains elusive (162).

Preliminary work utilising fluorescently labelled was performed to investigate the binding of P4 to cell surfaces and the possibility of the peptide being internalised within the cell (data in appendix). The experiments utilised flow

cytometry and confocal microscopy to investigate the fate of P4 during cell-peptide interactions and showed that P4 binds to cell surfaces and suggested that around 13% of P4 that binds to cells is internalised. As part of this project PK/PD studies were also attempted, using HPLC to measure P4 levels in plasma, these were unsuccessful with no P4 being detected in mice 3 minutes (shortest time it was feasible to inject P4, anaesthetise mouse and collect blood) after injection. The fact that the peptide binds to cell surfaces and that some it is subsequently internalised may explain why PK/PD measurements were unsuccessful.

Whether internalisation of P4 is necessary for P4 function is unknown. To better understand P4 function it would be important to know if P4 is entering cells and if so through what mechanism. P4 has an isoelectric point of 9, which makes it a cationic peptide. Cationic peptides can enter cells through a receptor independent manner as shown in Figure 85.

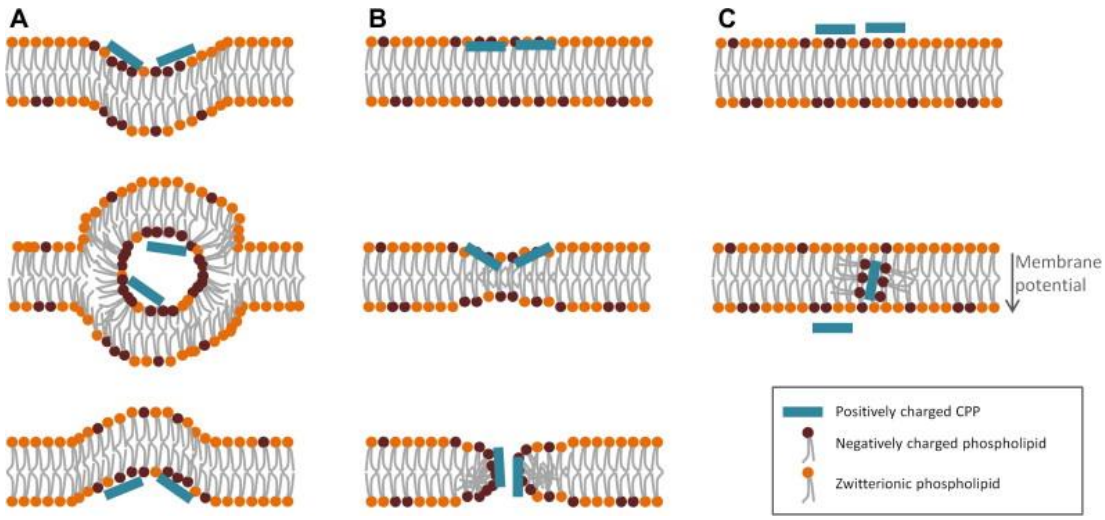


Figure 85 Direct translocation of cationic peptide
Examples of the proposed mechanisms for direct translocation. (A) Inverted micelle formation. (B) Pore-formation. (C) Adaptive translocation. From Bechara *et al*. 2013 (212)

Each of these models is dependent on the net positive charge of the peptide disrupting the normal functioning of the cell membrane (212). The confocal images of fluorescent P4 in macrophages however show the peptide in spherical bodies within the cell, which suggests that direct translocation of the peptide may not be the route through which P4 enters cells and that a form of endocytosis may be more likely and could be confirmed with assays utilising endocytosis inhibitors. The fact that these confocal images do not show entry of peptide into all cells should be investigated further to confirm that uptake is definitely occurring and whether internalisation of P4 is important to P4 function or if binding of P4 to the cell surface is sufficient for the peptide to take its effect.

The work performed so far with fluorescently labelled P4 (fP4) is preliminary and there are many more ways that fP4 could be utilised to answer questions about the mechanism of function of P4. One way it could be utilised is to investigate the fate of the peptide *in vivo*; using small animal imaging systems that can detect fluorescent compounds in live animals the peptide could be tracked within the mouse to answers questions such as do neutrophils treated with P4 show different patterns of migration within the mouse and how long these cells and the peptide itself persists.

As severe infections occur disproportionately in those under 5 years, the elderly and immune-compromised patients it is important that studies investigate the efficacy of P4 in these populations are performed. Aging is known to result in a reduction in neutrophil function whilst maintaining normal neutrophil numbers, so a treatment, which could possibly enhance neutrophil function, could be of great benefit to elderly patients (213).

Using P4 to treat fungal infections would also be of interest, severe fungal infections are increasing in prevalence and are an import cause of infection in ICUs (214). Given the difference in the way neutrophils process fungal hyphae compared with smaller pathogens it would be important to see if P4 had an effect of the interaction between neutrophils and fungi (93). Patients with fungal infections are also often immune-compromised; HIV infected patients, those receiving chemotherapy and recipient of solid organ transplants are at particular risk of fungal infection and antifungal drugs often have high host toxicity so alternative treatment options would be of great benefit to these patients (215, 216).

Appendix

A. Fluorescent P4 – Methods and Materials

Fluorescently labelled P4 peptide was purchased from Peptide Synthetics (UK). The peptide was synthesised with an additional lysine residue labelled with TAMRA added to the C-terminal.

1. Peptide binding

Mouse neutrophils were isolated from bone marrow and treated with fluorescently labelled P4 as described in Chapter V.B.2. Treated cells were centrifuged at 300 g to pellet cells, the supernatant was discarded and the cells incubated for 5 mins with either PBS or trypsin. Cells were pelleted gain, the supernatant discarded and resuspended in RPMI before analysing by flow cytometry.

2. Confocal imaging

Confocal studies used J774 macrophages as strongly adherent cells were needed, when attempted with neutrophils the cells were too loosely adhered to allow proper focusing of the microscope.

Macrophages were seeded into 35 mm glass bottom tissue culture dishes at a density of 10^6 cells per dish. After allowing to adhere for 2 hours cells were treated with fluorescently labelled P4 for 20 minutes. Cells were washed with PBS 3 times before imaging with LMS 510 multiphoton microscope.

B. Results

1. Fluorescent P4 – flow cytometry

What happens to P4 once it comes into contact with cells has not previously been studied. The following experiments were designed to explore the fate of P4 after contact with cells. Cells were washed with trypsin to remove surface bound peptide from cell before flow cytometry to establish whether P4 is entering cells or remains bound to cell surfaces.

The top pane of Figure 86 shows fluorescence levels in neutrophils treated with fluorescently labelled P4, which have been washed in either PBS or trypsin. The bottom pane shows fluorescence levels in neutrophils treated with non fluorescent P4 and neutrophils treated with fluorescent P4 then washed in trypsin. Washing with trypsin led to a significant reduction in the fluorescence of neutrophils treated with fluorescent P4 (p=0.008). However the levels of fluorescence in trypsin treated neutrophils was still significantly higher than those treated with non fluorescent P4 (p=0.0313). Levels of fluorescence in trypsin washed cells were 13% of the levels of neutrophils washed in PBS. This suggests that some of the fluorescence could not be removed by the trypsin and perhaps had been internalised with the cells.

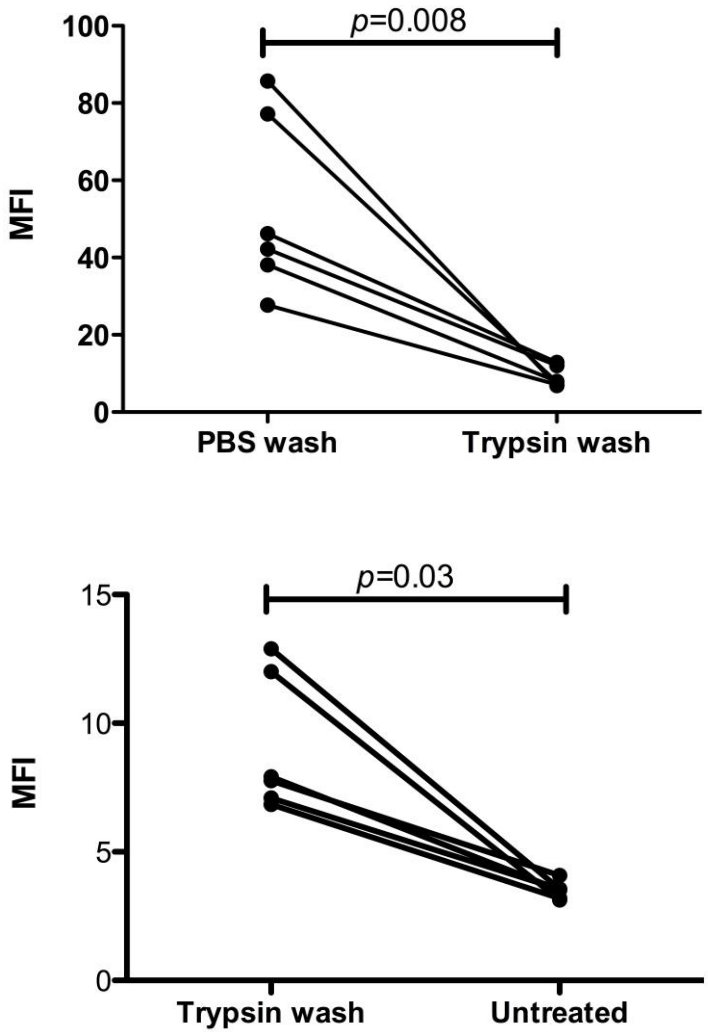


Figure 86 Binding of fluorescent P4 to naïve mouse bone marrow neutrophils
The bone marrow from 6 mice was collected and neutrophils isolated, neutrophils were treated with fluorescent P4, normal P4 or DEPC treated water for 1 hour. Cells were then washed in either trypsin or PBS and the fluorescence of cells measured by flow cytometry. Top: Fluorescent P4 -PBS washed vs. trypsin washed. Bottom: Cells treated with non fluorescent P4 vs. fluorescent P4 washed in trypsin. Analysed by paired t-test top p=0.008 and bottom p=0.03

2. Fluorescent P4 – confocal imaging

Imaging of cells treated with fluorescent P4 was performed to further establish the fate of P4 when it comes into contact with cells.

Figure 87 shows images of J774 macrophages treated for 20 mins with fluorescently labelled P4. Distinct spherical areas of fluorescent P4 (red) can be seen with the cells.

Figure 88 shows sequential Z-stacks at a distance of 1 μm between each image from A-D. The appearance of P4 (red) in sequential cuts through the cell suggests that the fluorescence is internalised within the cell.

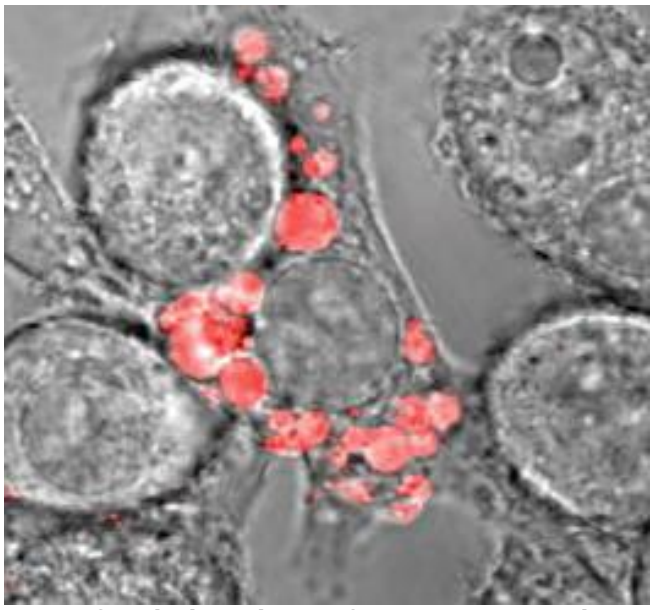


Figure 87 Live confocal imaging of J774 macrophages treated with fluorescently labelled P4

J774 macrophages were treated with P4 peptide for 20 mins, the cells were then washed with PBS before being imaged. P4 is shown in red.

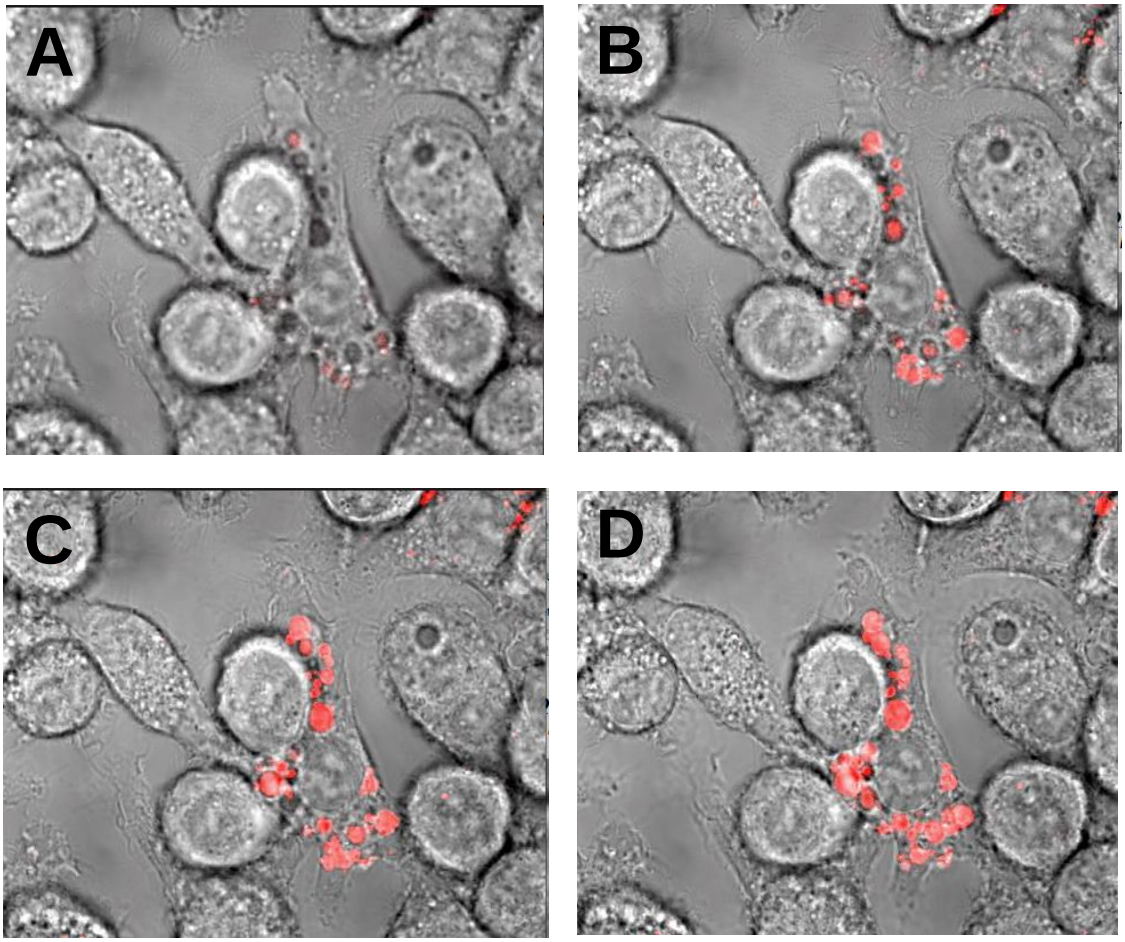


Figure 88 Live confocal imaging of J774 macrophages treated with fluorescently labelled P4 – Z stacks

Live confocal image of J774 macrophage treated for 20 mins with fluorescently labelled P4 (red). Images A-D show sequential Z-stacks 1 μm apart.

C. Discussion

Bone marrow neutrophils from naïve mice bound P4 peptide as evidenced by increases in fluorescence in cells treated with fluorescently P4, 87% of this fluorescence was removed by treatment with trypsin but levels of fluorescence were still significantly higher than in cells treated with non-fluorescent P4. This suggests the around 13% of the peptide may be internalised by the cells.

Images of J774 macrophages suggest that the P4 peptide is internalised by cells within 20 mins of treatment with P4 peptide. It is noted however that not all cells within frame showed uptake of P4 peptide.

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