Complement-mediated neutrophil dysfunction in critical illness

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DECLARATION

The work described in this thesis is my own unless otherwise acknowledged. No part of this work has been submitted in candidature for another degree.

Andrew Conway Morris
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This work is dedicated to my wife, Anna and two children Felix and Nils, the best family anyone could wish for.

Abstract

Critical illness, constituting an acute illness or injury resulting in organ dysfunction and failure, is associated with a profound, systemic activation of the immune system and inflammation-mediated organ damage. However, critically ill patients suffer a high rate of nosocomial infection with secondary sepsis being a common cause of death. This high prevalence of secondary infections argues for the influence of an immune suppression that may, at first glance, appear paradoxical in light of the pro-inflammatory nature of critical illness. Although immune cell hypo-function has been noted in clinical and experimental critical illness, the mediators of these effects remain poorly defined. In this thesis, neutrophil function was examined in the context of clinically suspected ventilator-associated pneumonia, a common and lethal intensive care acquired infection (ICU-AI). This demonstrated impaired bactericidal functions (phagocytosis and reactive oxygen species production) in neutrophils from both the peripheral and pulmonary compartments; however there was ample evidence of co-existent neutrophil activation (both cell surface markers and soluble mediators) and inflammation. An investigation of possible mediators of neutrophil dysfunction revealed a major role for C5a, the pro-inflammatory anaphylotoxin derived from complement C5. Recombinant C5a applied to healthy donor neutrophils was able to drive the defect in phagocytosis by phospho-inositol 3 kinase delta-mediated inhibition of RhoA and subsequent down regulation of actin polymerisation. The defects in RhoA and actin function were reversible with granulocyte-macrophage colony stimulating factor (GM-CSF) applied *ex vivo*, restoring phagocytic function to normal. Similar defects in RhoA and actin, and effective treatment with GM-CSF, were found in neutrophils from critically ill patients. In a second cohort of critically ill adults recruited prior to developing any ICU-AI, C5a-mediated neutrophil dysfunction was an independent predictor of acquiring nosocomial infection, being associated with a 5.4 fold increased risk (95% Confidence interval 1.4-21.0). The same cohort of patients also displayed two other features of immune suppression, namely monocyte deactivation and elevated proportions of regulatory T-cells that were also associated with increased risk of infection (relative risk of infection 3 (95%CI1.3-6.9) and 2.4 (95%CI1.3-4.2) respectively). These measures acted additively with C5a-mediated dysfunction, those with no immune impairment having a zero rate of nosocomial infection with cumulative increases in impairments being associated with a progressive increase in risk of infection (p=0.0004 by Chi squared for trend). In conclusion, critical illness is characterised by a complex inflammatory state with features of simultaneous hyper- and hypo-activation. This remarkable duality is illustrated by the ability of a pro-inflammatory molecule, C5a, to drive neutrophil dysfunction, with this dysfunction being associated with a serious adverse event –nosocomial infection.

Abbreviations

AC	Adenylate cyclase
ALI	Acute lung injury
AM	Alveolar macrophage
ANOVA	Analysis of variance statistical test
APACHE-II	Acute physiology and chronic health evaluation, second iteration
ARDS	Acute respiratory distress syndrome
BSI	Blood stream infection
BAL	Broncho-alveolar lavage
BALF	Broncho-alveolar lavage fluid
C3a	Anaphylotoxin formed by cleavage of complement component C3
C5a	Anaphylotoxin formed by cleavage of complement component C5
cAMP	Cyclic adenosine mono-phosphate
CARS	Compensatory anti-inflammatory response syndrome
CD	Cluster of differentiation
CD4	Cluster of differentiation 4
CD25	IL-2 receptor
CD88	C5a receptor type 1 (also denoted C5aR1)
CGD	Chronic granulomatous disease
CRBSI	Catheter-related blood stream infection
CVC	Central venous catheter
DC	Dendritic cell
'desarg'	Metabolite of C3/5a formed by carboxypeptidase removal of terminal arginine
DIC	disseminated intravascular coagulation
DMEM	Dulbecco's modified Eagle's medium
DO_2	Delivery of oxygen
EDTA	Ethylenediaminetetraacetic acid
ERK	Extra-cellular signal-regulated kinase
ETT	Endo-tracheal tube
FCS	Fetal calf serum
fMLP	formyl methionyl leucyl phenylalanine (also abbreviated to fMLF)
FOXP3	Forkhead box protein 3
GM-CSF	Granulocyte-macrophage colony stimulating factor
HAI	Hospital-associated infection
HAP	Hospital-associated pneumonia
HLA-DR	Human leucocyte antigen-DR
HIV	Human Immunodeficiency virus
HNE	Human neutrophil elastase
HV	Healthy volunteers (not age/sex matched cf Matched Volunteers)
IC_x	Inhibitory concentration X, where x denotes the reduction in activity relative to unihibited enzyme
ICU	Intensive care unit
ICU-AI	Intensive care unit associated infection
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-8	Interleukin 8 (also denoted CXCL8)
IL-10	Interleukin 10

INF-γ	Interferon gamma
IPEX	Immune dysregulation polyendocrinopathy enteropathy X-linked syndrome
IQR	Inter-quartile range
LAD	Leucocyte adhesion deficiency
LPS	Lipopolysaccharide
LTB4	Leukotrine B4
MARS	Mixed anti-inflammatory response syndrome
MIF	Macrophage migratory inhibitory factor
MODS	Multi-organ dysfunction syndrome
MV	Age and sex matched volunteers
NADPH	Nicotinamide adenine dinucleotide phosphate
NG	Naso-gastric
PAF	Platelet activating factor
PBN	Peripheral blood neutrophil
PI3K	Phosphatidylinositol 3-Kinase
PKA	Protein Kinase A
PKB	Protein Kinase B
PKC	Protein Kinase C
PR3	Proteinase 3
rhC5a	Recombinant human C5a
ROC	Receiver-operator characteristic
ROS	Reactive oxygen species
sCD62L	Soluble CD62L, also known as L-selectin
SOFA	Sequential organ failure assessment score
SARS	Severe acute respiratory syndrome
SAPS	Simplified acute physiology score
SDD	Selective digestive decontamination
SIRS	Systemic inflammatory response syndrome
SSI	Surgical site infection
SVO_2	Mixed venous oxygen saturation
T-cell	Thymic matured lymphocyte
TLR	Toll-like receptor
T-reg	Regulatory T-cell (CD4+CD25+FOXP3+)
TNF-α	Tumour necrosis factor alpha
TPN	Total parenteral nutrition
UTI	Urinary tract infection
VAP	Ventilator-associated pneumonia
VILI	Ventilation-induced lung injury

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Article I. Introduction and literature review

Section 1.01 Chapter overview

This chapter will present the background to the thesis, setting out the background to the hypotheses, which will be pursued in subsequent chapters. First is a description of the problems of nosocomial infections in intensive care units, and reasons for suspecting a role for specific host factors in their acquisition. Then follows a discussion of such potential host factors and an illustration of the issues through an exemplar infection, ventilator-associated pneumonia. Next is an exposition of the concept of 'critical illness', representing a common final pathophysiological pathway characterised by systemic immune activation. The apparently paradoxical finding of concurrent immune dysfunction will be presented, with discussion of the current state of knowledge in this field. This is followed by a discussion of two crucial aspects of physiological immune function; namely neutrophil functions (with specific reference to phagocytosis) and the complement cascade, and their role in killing invading microorganisms. Finally I will set out my principal hypotheses, which will be tested in the subsequent chapters.

Section 1.02 Nosocomial infection

(a) Epidemiology of hospital- and intensive care-associated infections

Hospital-associated infections (HAI) have been recognised as a cause for concern for a considerable period, being highlighted by 19th century doctors including Ignaz Semmelweis (1847) and Joseph Lister (1867), and remain a significant issue in many healthcare systems in the 21st century. The concerns arise from several considerations. First, such infections commonly involve multi-resistant organisms (McGowan, Tenover 2004) that require potent, potentially toxic anti-microbial therapy (Davey, Marwick 2008). Second, these infections are associated with increased rates of mortality, morbidity, duration of admission and costs (Vincent 2003). Thirdly, there is a belief that such infections represent an avoidable form of iatrogenic harm (Bailey, Ries 2005).

Defining the exact nature of the HAI problem remains problematic. Although a convention exists to define only those infections occurring 48 hours or more after admission as 'hospital-associated' (Suetens et al. 2007, American Thoracic Society, Infectious Diseases Society of America 2005), different studies and organisations use different diagnostic criteria (Suetens et al. 2003). This results in considerable variability in reported rates of infections between studies, units and countries that significantly limits the ability to undertake comparative analyses. Simply changing the method of obtaining microbial cultures can alter rates of diagnosis of diseases such as ventilator-associated pneumonia (VAP) (Conway Morris et al. 2009)

However, these issues notwithstanding, clear differences exist between certain groups of patients. Estimates of the prevalence of nosocomial infection amongst general medical and surgical patients range from 4-6% (Pellizzer et al. 2008, Reilly et al. 2008). In striking contrast, amongst patients requiring organ support in intensive care units (ICU), the prevalence rises to 25-40% (Vincent 2003, Vincent et al. 1995). These latter rates are similar to those found in neutropaenia (GIMEMA investigators 1991, Schimpff et al. 1978). Although attributing direct mortality to nosocomial infection remains problematic (see below), one infection alone, VAP, has an estimated mortality of 20-30% (Chastre, Fagon 2002). Each episode prolongs ICU stays by 4.5 days (Chastre, Fagon 2002) and adds $40,000 in costs (Baker, Meredith & Haponik 1996). This impact has led to concerted efforts to prevent and even eradicate infections such as VAP and catheter-related blood stream infections (CRBSI) from ICUs (Institute for Health Care Improvement 2005 a, Scottish Intensive Care Society Audit Group, 2008).

(b) Microbiology of ICU associated infections

The microbiology of nosocomial infections in general, and ICU-associated infections (ICU-AI) in particular, provokes further questions. ICU-AIs are caused by a variety of organisms. Sixty-two per cent of infections are caused by Gram-negative bacteria, 47% Gram-positive, and 19% by fungi, with anaerobes and viruses making up a small number (Vincent et al. 2009). As some infections are polymicrobial the percentages quoted are >100% in

total. A comparison of the organisms found in ventilator-associated pneumonia compared with severe (i.e. ICU-managed) community-acquired pneumonia is shown in Table I-1 below, indicating marked differences both in categories of organisms and the specific species. Many of the VAP organisms exhibit both intrinsic and acquired resistance to antimicrobials, reflecting the selective pressures exerted by the widespread use of such drugs (Jones 2010).

	Ventilator-associated	Severe community-acquired
Gram-positive	**32%**	**54%**
Staphylococcus aureus	20% (MRSA 11%)	5%
Coagulase negative *Staphylococcus*	1%	0.2%
Streptococcus pneumoniae	4%	46%
Other *Streptococcus* spp.	8%	3%
Gram-negative	**65%**	**26%**
Enterobacter spp.	14%	6%
Pseudomonas aeruginosa	24%	5%
Haemophilus spp.	10%	11%
Acinetobacter spp.	8%	0.2%
Neisseria spp.	3%	0.2%
Stenotrophomonas maltophilia	2%	0%
Other Gram–negative	4%	4%
'Atypical' bacteria	0%	8%
Anaerobes	1%	0.2%
Fungi	1%	0%

Table I-1: Common organisms involved in ventilator-associated and severe community-acquired pneumonia.
Numbers are derived from 24 studies of VAP (reviewed by (Chastre, Fagon 2002) and 4 studies of severe (i.e. requiring ICU admission) community-acquired pneumonia (Wilson, Ferguson 2005, Rello et al. 2003, Ruiz et al. 1999, Moine et al. 1994). Remaining percentage made up of other low frequency organisms including viruses. MRSA - Methecillin resistant *Staphylococcus aureus*.

Although the precise nature of the infecting organisms and their resistance patterns are undoubtedly influenced by local environmental prevalence and antimicrobial use (Kates et al. 1991, van Loon et al. 2005), this alone cannot explain the differences between hospital/ICU-associated and community-associated infections. First, it is notable that, despite being exposed to the same microbial ecology, hospital staff seldom acquire infections from such organisms,

despite frequently being vectors (Morgan et al. 2010, Mermel et al. 2003) and carriers (Whitman et al. 2008, Suffoletto et al. 2008). The experience of novel viruses such as influenza A/H1N1 2009 and the SARS coronavirus (Cheng et al. 2010, Lai, Yu 2010) demonstrate that hospital staff can and do acquire infections from their work environment. Second, at least some of the organisms responsible for ICU-AIs such as coagulase-negative Staphylococci and *Candida albicans* are, in immuno-competent adults, seldom more than commensals.

The high prevalence of nosocomial infections and the microbiological features noted above suggest that there are specific host factors involved in their acquisition. The great majority of ICU patients do not suffer from classic immunocompromise such as that produced by human immunodeficiency virus (HIV) infection, neutropaenia or immunosuppressive drugs. However, the rates of nosocomial infection are similar to those described in such patients, thus pointing towards less well-recognised and less overt immune dysfunction.

(c) Risk factors for intensive care-associated infection

Studies by various groups have identified a variety of factors that are associated with increased risk of acquiring infections within the ICU (Vincent 2003, Pellizzer et al. 2008, Al-Rawajfah, Stetzer & Hewitt 2009, Gastmeier et al. 2007). These can be broadly divided into intrinsic and extrinsic factors, as listed in Table I-2 below.

Extrinsic factors	Presence of invasive devices (e.g. ET tube, CVC, urinary catheters, NG tube)
	Prior use of broad-spectrum antimicrobials
	Immunosuppressive therapies including corticosteroids
	Renal replacement therapy
	Blood transfusion
	Drugs (stress ulcer prophylaxis, sedatives, paralysing agents)
	Recumbent position
	Parenteral nutrition
	Poor dental hygiene/oral flora
	Tracheostomy
Intrinsic	Age
	Sex
	Pre-existing co-morbid conditions, e.g. structural lung disease or diabetes mellitus
	Type of presenting illness – e.g. burns or major trauma
	Severity of precipitating illness
	Lifestyle factors (smoking, alcohol, illicit drug use)
	Nutritional status
	Immune dysfunction - both pre-existing and ICU-acquired

Table I-2: Factors associated with nosocomial infection in ICU.
ET-endotracheal, CVC-central venous catheter, NG-nasogastric.

The putative mechanisms by which these risk factors exert their influence are subject to debate, however the reasonable suppositions are as follows. The placement of foreign bodies, such as endotracheal tubes, indwelling central venous devices and urinary catheters breach the physical defences of skin and mucous membranes and provide nidi for microorganisms to colonise and hence infect (Passerini et al. 1992). This is supported by the relationship between

organisms found to be colonising that later cause infection (Kerver et al. 1988, Luft et al. 2010), and the effectiveness of aseptic insertion (Raad et al. 1994) and antimicrobial coatings on reducing device associated infections (Wang et al. 2010, Kollef et al. 2008b, Karchmer et al. 2000). Organisms themselves contribute by changing phenotype, and forming biofilms that are resistant to antimicrobial penetration and immune cell attack (Singh et al. 2010, Fux et al. 2005). However it should be noted that many non-critically ill patients also have indwelling devices, such as tracheostomy tubes for home ventilation or long-term vascular access devices and do not experience the same rate of infections (Splaingard et al. 1983, Morar et al. 1998, Jean et al. 2001). The requirement for endotracheal intubation, central venous catheter insertion and urinary catheterisation is also associated with severity of illness (and in the case of endotracheal intubation, sedation and analgesia), so additional influences are undoubtedly present beyond the simple existence of a foreign device.

Broad-spectrum antimicrobials are also associated with nosocomial infection. This association is in part due to their common use in hospital environments, where infections are acquired, as well as with their effects on commensal flora allowing colonisation, overgrowth and frank infection by more pathogenic organisms (McNulty et al. 1997, Scannapieco, Stewart & Mylotte 1992). Broad-spectrum antimicrobials also worsen the severity of infection by inducing resistance and selecting out organisms that have already developed resistance (Trouillet et al. 1998, Barza et al. 1987), so making infections harder to treat, necessitating higher doses of more toxic antimicrobials. However several groups of patients perceived to be at higher risk of infection, such as those with burns or

fulminant hepatic failure, commonly receive prophylactic antibiotics with evidence of reduced infections and improved survival (Avni et al. 2010, Rolando et al. 1993). Indeed a systematic review of antibiotic prophylaxis for respiratory infections in critically ill patients found a survival benefit and reduced infections for combined systemic and topical antimicrobials (D'Amico et al. 1998).

What is more certain is the finding of oropharyngeal colonisation preceding the occurrence of VAP caused by the same organisms (Bonten et al. 1996), with micro-aspiration past the cuff of the endo-tracheal tube being the most likely route of infection (reviewed in Estes, Meduri 1995). Enhanced dental hygiene (Sona et al. 2009), oral decontamination with chlorhexidine (Garcia et al. 2009), selective digestive decontamination (SDD) (Schultz, Haas 2011) and endotracheal tubes with subglottic suction and improved cuff seal (Bouza et al. 2008) have demonstrable benefits on VAP, supporting this as a significant factor.

Renal replacement therapy is also associated with nosocomial infection, not only secondary to invasive devices required for dialysis or haemofiltration but also due to the effects of renal failure and uraemia on immune cell function (Kato et al. 2008). Interestingly, in end-stage renal disease at least, there appears to be concomitant hypercytokinaemia/inflammation and immune dysfunction. (Stenvinkel et al. 2005) Despite recent improvements in dialysis and filtration membranes, extracorporeal circulation can result in immune cell and protein activation, including complement activation (Royston 1997).

Blood transfusion remains an area of considerable controversy in critical care (Walsh, Wyncoll & Stanworth 2010), with only a single randomised trial published (Hébert et al. 1999) and conflicting data from epidemiological studies (Vincent 2002, Vincent et al. 2008). Blood transfusion is linked to length of stay in the ICU (Vincent 2002) and can occur at various time points throughout the stay, both of which may confound any specific link with nosocomial infection. However heterologous blood is linked to immune depression (Triulzi et al. 1992) and therefore could be mechanistically linked to nosocomial infection, although this relationship is less clear in the post-leukodepletion era (Jensen, Hokland & Nielsen 1996).

Amongst the various drugs given to ICU patients, several classes have been linked to nosocomial infection. For obvious reasons drugs used as immunosuppressants have the clearest links to nosocomial infection (Ylipalosaari et al. 2006), with this also being the case for 'low'/'replacement' dose steroids in septic shock (Sprung et al. 2008). Perhaps less obviously several other classes of drugs are also associated with nosocomial infection. Antacids such as H_2 antagonists and proton pump inhibitors have a significant effect on reducing stress ulcers and upper gastro-intestinal (UGI) bleeding, but are associated with an increased risk of pneumonia (Beck-Sague et al. 1996). This is likely to be related to increased bacterial colonisation of stomachs with alkaline content (Forster et al. 1982). Critically ill patients, even without antacid therapy, have alkaline stomach fluid resulting from failure to secrete H^+ ions (Higgins, Mythen & Webb 1994). The gastric stasis and gut failure, which is

commonly found in critical illness, can further exacerbate this, providing a reservoir for bacterial growth and aspiration (Fruhwald, Holzer & Metzler 2008). Sedatives and paralysing agents have also been linked to an increased risk of nosocomial infection. This is thought to relate to the effects these have on duration of mechanical ventilation, ICU stay and impairment of protective reflexes such as coughing (Quenot et al. 2007). Some agents used, such as propofol and opioids, have direct effects on immune cell function (Huttemann et al. 2006, Clark et al. 2007) although the relevance of this is uncertain (Rittner, Roewer & Brack 2010). However there may be non-mechanistic explanations for the association, as limiting sedation results in reduced ventilation and reduced rates of VAP (Quenot et al. 2007).

Amongst other drugs used in critical illness, several have noted immunomodulating activities. Beta-adrenoceptor agonists, including catecholamines and synthetic selective agonists such as dobutamine, can impair neutrophil functions (Ignarro, Lint & George 1974) as well as altering lymphocyte and dendritic cell functions (reviewed in Montmollin et al. 2009). Aside from any antimicrobial activity, macrolide antibiotics such as clarithromycin can reduce neutrophil transmigration and reactive oxygen species production (Akamatsu H et al. 1996). The HMG-CoA reductase inhibitors, known collectively as statins, also have demonstrable immunomodulatory effects (Weant KA & Cook AM 2007) that are currently under investigation as therapeutic options in critical illness, and significant numbers of patients admitted to the ICU are on statin therapy prior to admission (O'Neal HR Jr et al.

2011). However to date none of these immunomodulatory effects have been shown to influence the acquistion of nosocomial infection within the ICU.

Total parenteral nutrition (TPN), with its high lipid load, tendency to cause hyperglycaemia, requirement for administration via a central vein and large external reservoir of a medium supporting microbial growth involves a variety of risk factors for nosocomial infection and has shown up in several epidemiological studies as an associated factor (Chow et al. 2008, Chen et al. 2006).

Tracheostomy remains a controversial risk factor for nosocomial infection, with studies conflicting as to its effects. Some indicate an increased risk (Ibrahim 2000), where others demonstrate more rapid weaning, lower sedation requirements and hence a reduction in ventilator-associated pneumonia (Moller et al. 2005).

Both TPN and tracheostomy tend to occur in patients remaining in ICU for longer periods of time, which may confound any relationship with nosocomial infection. Furthermore infections themselves often prolong ICU stay, leading to a requirement for such interventions (as with other factors such as blood transfusion). A failure to account for the temporal relationship between ICU-AI and these interventions (i.e. ensuring that they occur *prior* to infection) could also lead to erroneous conclusions about the direction of causation.

Intrinsic patient factors make up the second group of variables associated with nosocomial infection. Pre-morbid health, and co-morbid conditions have a significant impact on outcomes from intensive care (Hofhuis et al. 2007), with an impact on physiological reserve and potential for recovery. Some factors have a specific association with nosocomial infection, including structural lung disease and diabetes (Celis et al. 1988). These associations extend beyond the intensive care unit and probably reflect both physical factors, such as abnormal lung anatomy allowing pooling of secretions and impaired clearance (Jeffery 1998), and impaired immune responses secondary to these diseases (Arcavi, Benowitz 2004, Turina, Fry & Polk 2005).

As will be discussed below, critical illness can arise from a variety of precipitating illnesses, which tend to produce a stereotyped immune response irrespective of the pathogenic origins (Bianchi 2007). However certain underlying illnesses do appear to bring a greater risk than others. Burns (Wurtz 1995) and trauma (Vincent et al. 1995) are both associated with an increased risk of nosocomial infection. Burns involve a profound inflammatory response (Bloemsma et al. 2008), as well as disruption of the protective barrier of the skin. Why trauma should be a risk factor for HAI remains a moot point, but it appears to remain an independent risk factor even when increased device utilisation, organ failures and illness severity are accounted for (Vincent et al. 1995).

The severity of the presenting illness, often assessed by scores such as the Acute Physiology and Chronic Health Evaluation (APACHE) Score, Sequential Organ Failure Assessment (SOFA) Score and Simplified Acute Physiology Score

(SAPS), is also associated with nosocomial infection. Disease severity itself cannot cause infections, but it is associated with the requirements for interventions noted above, as well as resulting from multi-organ dysfunction which is the hall-mark of severe systemic inflammatory response syndrome (SIRS) and critical illness (see below). It appears likely that immune failure represents another organ system failure (see below). It should be noted that several large studies (Vincent et al. 1995, Craven et al. 1988) did not demonstrate an independent effect of severity of illness once other factors had been corrected for.

Tobacco smoke has a widely acknowledged deleterious effect on a range of body systems, including the lungs and heart. Smoking is a well-established risk factor for pulmonary and extra-pulmonary infections, due to its effects on mucociliary clearance and immunomodulatory impact (Sopori 2002). This risk also extends into nosocomial infections in the ICU (George et al. 1998), although this may to some extent reflect long-term health damage from smoking in addition to acute tobacco toxicity (Delgado-Rodriguez et al. 2003).

Alcohol similarly has a direct immunotoxic effect (Zambell et al. 2004, Nilsson et al. 1996), and is also linked in epidemiological studies to nosocomial infection (Gacouin et al. 2008). Once again the long-term damage induced by excess alcohol on multiple organ systems may play as significant a role as the acute impact on immune cell function. Intravenous drug use, perhaps surprisingly, is not a widely reported risk factor for HAI, although it is associated with HIV

infection and acquisition of multi-drug resistant *Pseudomonas* infections (Tacconelli et al. 2002).

Malnutrition is a predictor of a variety of complications of intensive care, including prolonged length of stay, delayed weaning and critical illness myoneuropathy (Giner et al. 1996). Each of these can result in increased risk of nosocomial infection, which is associated with malnutrition in epidemiological studies (Schneider et al. 2004). Determining the immunological effects of malnutrition in humans can prove difficult, as infections can result in malnourishment as well as *vice versa*. In addition malnourishment is a heterogeneous collection of conditions ranging from protein-energy malnutrition through to specific absence of key macro- or micronutrients. Anorexia nervosa reflects a fairly 'pure' malnourishment, with defects found in lymphocyte function that reverse on re-feeding (Allende et al. 1998). Interestingly results from the per-protocol subgroup of the SiGNET trial of selenium supplementation of TPN revealed a 10-15% reduction in nosocomial infection (Andrews et al. 2011).

Specific immune defects may arise prior to ICU admission, or during it. As noted above a variety of drugs and similar treatments used in ICU have immunomodulatory effects, as may pre-admission factors such as smoking, alcohol and nutritional status.

There are a number of acquired and in-born diseases of immune function, including HIV infection, haematological malignancies and associated cytopaenias, and 'primary' immunodeficiency arising from congenital and genetic defects (of which approximately 200 have now been described) (Casanova, Abel 2007). These diseases are associated with acquisition of infection, both in the community and in hospital. However it is suggested that critical illness itself is associated with, and indeed generates, a state of immuno-suppression, which may prove to be one of the most important factors in the development of nosocomial infection in ICU.

The above points about ICU-AI will now be discussed in the context of a common and serious infection, ventilator-associated pneumonia (VAP). A section will then follow this on the concept of critical illness and finally a review of the current state of knowledge on critical illness-induced immune defects.

Section 1.03 Ventilator-associated pneumonia, an exemplar nosocomial infection

Infections of the respiratory tract form the commonest group of infections found in the intensive care unit (Vincent et al. 1995, Vincent et al. 2009). Of the nosocomial infections VAP is the commonest (Vincent et al. 1995), occurring in between 8-30% of ventilated patients (Chastre, Fagon 2002). The prevalence of VAP is influenced by variations in diagnostic technique (Conway Morris et al. 2009), as well as varied patient and staff factors (George et al. 1998, Blot et al. 2011). The issue of VAP diagnosis, and hence prevalence, has become further

complicated by the use of VAP rates as a 'care quality' indicator (Institute for Healthcare Improvement, 2005 b).

The risk factors associated with VAP are largely those identified above, especially those associated with the pulmonary system such as intubation, mechanical ventilation and micro-aspiration (reviewed in Chastre, Fagon 2002). Interventions to reduce these, such as using non-invasive ventilation to avoid intubation, head of bed elevation and sub-glottic suction have demonstrated reductions in VAP rates (reviewed in Rello et al. 2010).

Pneumonia is, strictly speaking, a histological diagnosis (Rouby et al. 1992), however this is seldom achievable *ante-mortem*, especially in mechanically ventilated patients who would tolerate open lung biopsy poorly. In the absence of a tissue sample, pneumonia is diagnosed on a combination of clinical, radiological and laboratory criteria. Although specific criteria vary from source to source, all include the requirement for evidence of inflammation (e.g. elevated or suppressed white cell count, elevated or suppressed temperature, acute phase proteins or tachycardia/tachypnoea), clinical pulmonary signs (e.g. muco-purulent sputum, auscultatory findings, impaired gas exchange) and radiological evidence of new (and persistent) consolidation, usually by plain radiograph or computed tomography (CT). This would then constitute 'clinical' or 'suspected' VAP. Most diagnostic criteria then look to identification of an infecting organism, either by direct culture or indirect methods such as antigen testing or serology. Some organisms, such as anaerobes and the 'atypical' respiratory bacteria, are difficult to culture, and others such as viruses are now routinely

detected by polymerase chain reaction (PCR) rather than complex cell culture methods used previously. The detection of organisms can be further complicated by inter-current antimicrobial therapy (Souweine et al. 1998), as conventional cultures can only detect live organisms. Those with positive microbiology are then generally termed 'confirmed' VAP. A diagrammatic representation of one of the diagnostic schemes, from the Hospitals in Europe Link for Infection Control through Surveillance (HELICS) program is shown in figure I-1. (HELICS 2004)

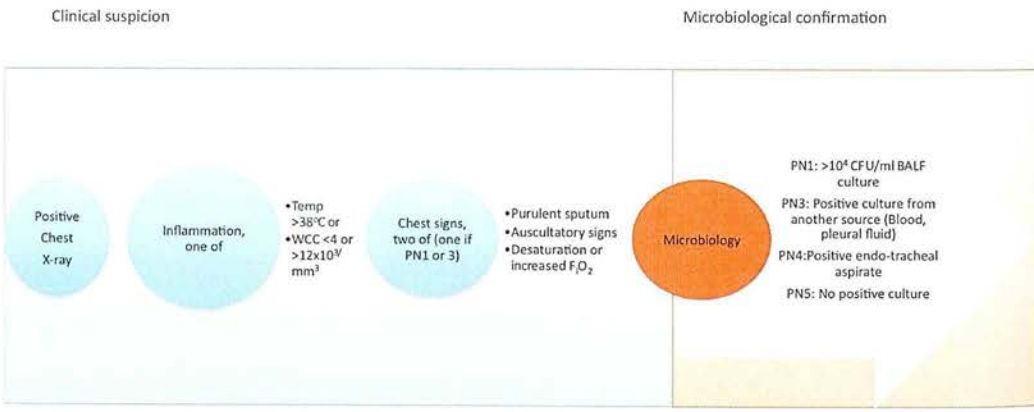


Figure I-1: HELICS method of VAP diagnosis.
The HELICS method requires clinical and radiological signs of pneumonia (left hand side) and microbiological sampling (right hand side). WCC-white cell count, CFU-colony forming unit, BALF-broncho-alveolar lavage fluid. 'Positive' chest x-ray being one with new alveolar shadowing consistent with consolidation. (HELICS 2004)

Mechanically ventilated patients present a number of problems in this diagnostic 'triad' of clinical inflammation, respiratory focus and radiological consolidation. The causes of inflammation in critically ill patients are legion, as discussed below, and a range of non-infective conditions can produce radiographic alveolar shadowing with associated lung signs. Acute lung injury (ALI) / acute respiratory distress syndrome (ARDS) in particular can not only mimic pulmonary infection, but can also be complicated by secondary infection in up to 60% of cases (reviewed in Chastre, Fagon 2002). In this setting, the addition of microbial cultures is vital for diagnosis rather than just guiding therapy. Many patients without clinical VAP have microbial colonisation of the endotracheal tube and trachea (Feldman et al. 1999). It is therefore not surprising that various studies suggest that use of endotracheal aspirate cultures produces a significantly higher rate of positivity compared to more protected sampling of the distal airways by broncho-alveolar lavage (BAL) or protected specimen brush (PSB). *Post-mortem* studies suggest that culture of organisms at 10^4 colony forming units (CFU)/ml from lavage fluid (and 10^3 CFU/ml from PSB samples) is associated with a histological diagnosis of pneumonia (Marquette et al. 1995), and hence these are widely used cut-offs for the diagnosis of VAP. However the use of invasive specimens for diagnosis and treatment remains restricted due to conflicting trial results (Fagon et al. 2000, Canadian Critical Care 2006), and the requirement for specialist skills and resources for bronchoscopy.

The effect of VAP on patient outcomes remains a hotly debated area. Although the consistent findings of prolonged mechanical ventilation, increased burden of

antibiotics and increased duration of stay suggest these are definite associations (reviewed by Chastre, Fagon 2002), the impact on mortality is less clear. Some studies suggest no impact on mortality, once other confounders are included (Papazian et al. 1996), while others suggest an independent increase (Vallés et al. 2007). Inappropriate or delayed antibiotics are associated with mortality, however, suggesting that these infections are not a benign epiphenomenon of critical illness (Iregui et al. 2002, Kollef et al. 2008a). One might expect the results of VAP prevention studies to shed some light on this area, however to date these reports have been too flawed to allow an effective judgement (Zilberberg, Shorr & Kollef 2009). Therefore at present the best estimate of attributable mortality increase in VAP is in the order of 20-30% (Chastre, Fagon 2002), but this remains an uncertain figure.

VAP therefore illustrates several of the issues around ICU-acquired infection. It is highly prevalent, presents a diagnostic challenge amongst the pro-inflammatory milieu of the critically ill patient and results in a significant burden of morbidity, pro-longed length of stay, antibiotic treatment and cost and probably also impacts on mortality. Although there are established risk factors, far from all patients exposed to such factors develop VAP and hence there is considerable scope to investigate the role of host factors such as immune suppression in VAP's causation.

Section 1.04 Critical illness: defining a concept

(a) Critical illness

Patients are admitted to intensive care units for a variety of reasons, and in a manner that will vary from institution to institution and country to country. For instance admission policies in the United States, where there are 20 ICU beds per 100,000 head of population, are likely to differ from the United Kingdom where there are only 3.5 beds per 100,000 (Adhikari et al. 2010). As a result one cannot simply define 'critical illness' as 'a requirement for intensive care admission', and yet many of the studies of ICU-acquired infection simply look at all admissions which may, in part, explain the apparent correlation between nosocomial infection rates and mortality seen between countries (Vincent et al. 1995, Vincent et al. 2009).

With the origins of critical illness care in intensive/critical care units arising from the invasive ventilation of patients during the 1953 Danish polio epidemic (Ibsen 1954), intensive care has focused on support for failing organ systems resulting from acute illness. Although various critical illness syndromes have been defined, such as sepsis (American College of Chest Physicians/Society of Critical Care Medicine 1992), ARDS (Bernard et al. 1994) and more nebulously 'multi-organ dysfunction syndrome' (MODS) (Marshall 2000), an overall, precise consensus definition of critical illness is missing (Adhikari et al. 2010, McLellan, McClelland & Walsh 2003). In the absence of this definition, it is generally accepted that 'critical illness' constitutes an acute illness resulting in organ dysfunction and failure. What constitutes 'organ failure' is a further area of debate, with definitions ranging from physiological parameters (Bellomo et al. 2004) to the requirement for exogenous organ support (Vincent et al. 1996). For

the purposes of this thesis, 'Critical Illness' is defined as an acute illness precipitating organ dysfunction severe enough to necessitate exogenous organ support.

(b) Pathogens, danger and inflammation

Critical illness is frequently associated with a systemic inflammatory response syndrome (SIRS), characterised by physiological manifestations of inflammation (altered temperature, leukocytosis or bone marrow suppression, tachycardia and tachypnoea). This is accompanied by biochemical and immunological evidence of immune system activation (Sakamoto et al. 2010, McILwain et al. 2010, Miyaoka et al. 2005). SIRS can arise from both infective and non-infective causes, with infective SIRS being referred to as sepsis, and associations with organ failure and shock further defining 'severe SIRS/sepsis' and 'SIRS/septic shock' (American College of Chest Physicians/Society of Critical Care Medicine 1992).

A range of microbial ligands, including lipopolysaccharide (LPS), peptidoglycan, flagellin and lipoteichoic acid, are collectively known as pathogen-associated molecular patterns (PAMPS). These are able to induce inflammation by activating toll-like receptors (TLRs), associated with activation of intracellular signalling pathways such as the nuclear factor kappa B (NF-KappaB) pathway, which transduce signals into an inflammatory response (reviewed by Beutler 2009). The activation of this response is crucial to the elimination of microbial invaders and the resolution of infection (Dennis et al. 2008). Until more recently, what was less clear was the mechanisms by which sterile insults such as

trauma, pancreatitis and ischaemia-reperfusion injury were able to stimulate a similar response. It is apparent that tissue damage, such as that arising from the above listed sterile insults, results in the release of endogenous molecules capable of eliciting similar responses to PAMPs. These endogenous molecules include high-mobility group box 1 (HMGB-1), a non-histone nuclear protein released following cell death and also by active secretion, which can activate NF-kappaB-dependent pathways via the receptor for advanced glycosylation end-products (RAGE) (reviewed in Sims et al. 2010). A variety of other such 'alarmins' have been identified, including heat shock proteins (HSP) (Wheeler et al. 2009), interleukin-1 alpha (IL-1a) (Joosten et al 2010), S100/calgranulin proteins (Ehrchen et al. 2009), cathelicidins such as LL-37 (Yu et al. 2007) and uric acid (Liu-Bryan et al. 2005), which can activate a variety of receptors including RAGE, TLRs and specific receptors such as IL-1R (Reviewed in Bianchi 2007). Further developments in the field of sterile inflammatory activation have come from the recent identification of released mitochondrial contents following trauma (Zhang et al. 2010a), which are capable of ligating formyl-peptide receptors. These also respond to similar ligands derived from bacteria, which are thought to share common ancestry with mitochondria prior to their inclusion in eukaryotic cells. It has been proposed that PAMPs and alarmins should be classified into a larger group, termed damage-associated molecular patterns (DAMPs) to indicate their common elicited response from the host (Bianchi 2007).

(c) Systemic inflammation

Whilst the concept of DAMPs, and their down-stream pathways, may indicate how inflammatory responses are triggered, how this leads to uncontrolled systemic inflammation, critical illness and multi-organ failure requires further explanation. The suggestion is of a progression from local inflammation at the site of injury/infection to systemic activation of inflammation, which if unchecked progresses to organ dysfunction (i.e. critical illness) and ultimately multi-organ failure (Matsuda, Hattori 2006).

How inflammation becomes uncontrolled and systemic remains a matter of debate. Although bacterial toxins, and bacteria themselves, can enter the circulation, bacteraemia is neither a pre-requisite nor a guaranteed precipitant of systemic inflammation and sepsis (Jones, Lowes 1996). Therefore outwith specific situations such as Staphylococcal toxic shock syndrome, systemic release of bacterial PAMPs may not be the crucial event. Several genetic polymorphisms have been associated with the development of severe SIRS and sepsis, including TLR-4 (Kumpf et al. 2010), tumour necrosis factor alpha (TNF-α) (Surbatovic et al. 2010), mannose binding lectin (Gordon et al. 2006) and IL-10 (Shu et al. 2003). It seems likely that differential regulation of the host response, by a combination of genetic and environmental factors, interacts with the initial insult to trigger the systemic activation of inflammation that characterises the early phases of sepsis and SIRS.

Defining the mediators involved in sepsis and SIRS, the sequence of events and the points at which adaptive inflammatory responses descend into maladaptive processes, are difficult, especially in human sepsis. Firstly it is rare to encounter

patients prior to their insult, and with most precipitating events even determining the time between onset and presentation can be problematic. Furthermore, events and mediators in some body spaces may not be reflected by changes in the blood (Conway Morris et al. 2010, Hart et al. 1986), although blood is the most commonly studied body fluid in critical illness. Investigators are therefore obliged to rely in part on data acquired from animal models, which are imperfect guides to diseases in humans (Mestas, Hughes 2004, Dyson, Singer 2009).

Animal models and human studies suggest a very early rise in pro-inflammatory molecules such as IL-1 and TNF-alpha, which in blood at least, tend to peak early (within minutes) before declining (reviewed inThijs, Hack 1995). Slightly later responses involve molecules such as IL-6 (Damas et al. 1992), monocyte chemotactic proteins 1 and 2 (MCP-1 and 2) (Bossink et al. 1995), IL-8 (Hack et al. 1992) as well as anti-inflammatory molecules such as IL-10 and the soluble TNF and IL-1 receptors (reviewed in Thijs, Hack 1995), alongside later mediators such as HMGB-1(Gibot et al. 2007). Interestingly, as predictors of outcome in sepsis, it appears that it is the magnitude of the anti-inflammatory response (as estimated by IL-10 concentrations) rather than the pro-inflammatory response (as estimated by TNF-alpha, IL-1, -6 and -8) that performs best (Gogos et al. 2000).

Several proteolytic plasma cascades activated in sepsis and SIRS are also implicated in the pathogensis of multi-organ failure. Specifically, sepsis and SIRS are accompanied by dysregulated, systemic activation of the complement, coagulation and fibrinolytic cascades (reviewed in Rittirsch, Flierl & Ward

2008). These pathways activate each other, so driving positive feedback mechanisms and exacerbating the damage and dysfunction (Amara et al. 2010a). The normal function of the complement cascade is discussed below (Section 1.08). However, in sepsis it undergoes massive activation with spillover of complement products into the systemic circulation, which results in a range of deleterious effects detailed in Table I-3 below, most of which are mediated by the anaphylotoxins and specifically C5a (Ward 2004). Almost all of this work is derived from animal models or *in-vitro* examination of human cells, rather than from patients with sepsis or SIRS.

Activated complement products, and C5a in particular, activate the coagulation system via tissue factor (Kambas et al. 2008) and stimulate the production of inflammatory cytokines such as HMGB-1 (Rittirsch et al. 2008). C5a is also directly chemo-attractive to neutrophils as well as stimulating the release of reactive oxygen species (ROS) (Jia et al. 2010) and lytic enzymes such as human neutrophil elastase (HNE) (Binder et al. 1999, Zeerleder et al. 2003). Both have been implicated in sepsis-associated organ damage and outcomes (Gardinali et al. 1992). Much as complement activation can activate the coagulation cascade, thrombin can also cleave C5 into C5a and C5b independent of its upstream pathways (Wetsel, Kolb 1983) (see section 1.08 below), so illustrating how these two systems can positively reinforce one another and contribute to their dysregulation and inappropriate systemic activation. The systemic activation of the coagulation system results in deposition of fibrin and formation of microthrombi in the microcirculation, and thus possibly contributes to the impaired tissue perfusion seen in sepsis (Zhou et al. 2003, Gaskins, Dalldorf

1976). Indeed, to date, the only therapy aimed specifically at sepsis-induced multi-organ failure, which has successfully completed phase three trials, is activated protein C, which targets the coagulation system amongst other effects (Bereczky, Kovacs & Muszbek 2010, Bernard et al. 2001). Concurrent activation of the fibrinolytic system results in the characteristic disseminated intravascular coagulation (DIC), which combines the microthrombotic problems noted above with a systemic coagulopathy and increased risk of haemorrhage (reviewed in Bick, Arun & Frenkel 1999).

Effect	Complement component(s) involved	References
Neutrophil recruitment[1]	C5a	(Shuster, et al. 1997)
Neutrophil activation[2], neutrophil elastase release[3]	C3/C5	(Zeerleder et al. 2003, Brekke et al. 2007)
Bacterial opsonisation[2]	C3b	(Brekke et al. 2007)
Direct bactericidal effects[1]	C5b-9 (Membrane attack complex)	(Flierl et al. 2008c)
Macrovascular dysfunction[1]	C5a	(Hugli, Marceau 1985)
Tissue factor activation/hypercoagulation[1]	C5a	(Laudes et al. 2002a)
Release of alarmins (e.g. HMGB-1)[1]	C5a	(Rittirsch et al. 2008)
Neutrophil dysfunction[1,2]	C5a	(Huber-Lang et al. 2002b)
Lymphocyte apoptosis[1]	C5a	(Riedemann et al. 2002)
Cardiac dysfunction[1]	C5a	(Niederbichler et al. 2006)
Hypothalamic-pituitary dysfunction[1]	C5a	(Flierl et al. 2009)

Table I-3: Effects of complement during sepsis.
1-animal models, 2-*in-vitro* studies, 3-human studies.

The various immune cells display altered functioning during sepsis and SIRS. Neutrophils containing proteolytic enzymes such as HNE and cathepsins (Boxer, Smolen 1988) as well as being a major source of ROS (Starr et al. 2011), typically become 'activated' by the soluble mediators found in sepsis including both endogenous molecules such as C5a and IL-8, and bacterial ligands such as LPS and formylated peptides. As noted above, mitochondria also express formylated peptides, releasing these upon tissue damage, and this may be one mechanism of neutrophil activation in sterile SIRS (Raoof et al. 2010). Although there remain discrepancies within the literature, it is generally accepted that sepsis and SIRS are accompanied by neutrophil activation (Sakamoto et al. 2010, Rosenbloom et al. 1995), so preparing them to fight actual or potential microbial invaders and remove damaged tissue (Borregaard 2010). However, high levels of activated neutrophils are linked to organ damage (Borregaard 2010) and failure as discussed below. Furthermore, in the setting of sepsis, neutrophils frequently display dysfunctions in key anti-microbial activities (see section 1.05 c below).

Monocytes and macrophages play a central role in co-ordinating immune responses, promoting neutrophil recruitment (Kreisel et al. 2010) and acting as antigen presenting cells to stimulate adaptive immune responses (Wolk et al. 2000). Monocytes exist in at least three sub-populations, each of which release specific profiles of cytokines and are relatively pro- or anti-inflammatory (reviewed in Ziegler-Heitbrock 2007). During systemic inflammation and sepsis these sub-populations alter in size, with expansion of the CD14+CD16+ sub-population (Fingerle et al. 1993). Interestingly, although this sub-population is

classically described as being 'hyper-inflammatory' (Ziegler-Heitbrock 2007), analysis of their function in patients with sepsis revealed *decreased* rather than increased TNF-α production (Horelt et al. 2002).

Macrophages can also exist in at least two different forms, termed M1 and M2, with M1 being predominantly pro-inflammatory and provoking a T_H1 response from T-lymphocytes, whilst M2 phenotypes promote inflammatory resolution and a predominant T_H2 response (reviewed inMege, Mehraj & Capo 2011). Sepsis is associated with alterations in the M1/M2 polarisation of macrophages; with the suggestion that unbalanced M1 activation is associated with poor outcomes (Benoit, Desnues & Mege 2008, Mehta et al. 2004).

T-lymphocytes are classically thought to be acted upon by the innate immune system, responding to stimuli from antigen presenting cells before either facilitating humoral or cytotoxic defence mechanisms. However in animal models of sepsis, there appears to be evidence of very early T-cell activation, which contributes to inflammation, innate immune activation and mortality (van Schaik, Abbas 2007). This is mirrored by findings of T cell activation in trauma (Walsh et al. 2000).

It must of course be noted that sepsis/SIRS do not simply affect immune cells, but also provoke dysfunction in a variety of other tissues including endothelium, muscle and secretory cells. Endothelial cells specifically play a role in initiating and amplifying inflammation with both PAMPS and alarmins 'activating' epithelial cells (reviewed in Aird 2003), resulting in initiation of coagulation,

inhibition of fibrinolysis (Moore et al. 1987) and increased immune cell adhesion and activation (Bauer et al. 2000, Katayama et al. 2000). Further endothelial responses include increased permeability and vasomotor relaxation, provoking the hypotension, tissue oedema and vascular stasis seen in sepsis (reviewed in Aird 2003).

(d) Mechanisms of multi-organ failure

It has been established above that critical illness is largely defined by systemic immune activation, however the second crucial part of the definition is the presence of organ dysfunction and failure. During critical illness, impairment can occur in any body system, however the predominant ones seen clinically are the respiratory, cardiovascular, renal, hepato-gastrointestinal and cerebral systems (Vincent et al. 1996). More recent work has identified failures in neuro-endocrine regulation (Beishuizen, Thijs 2004), muscular function (Callahan, Supinski 2010) and, most pertinently to this thesis, the immune system (van der Poll, Meijers 2010).

Considerable debate continues as to the causes of organ failure in critical illness. There are several competing theories, which can be broadly grouped into 'inflammatory organ damage', 'gut-driven', 'tissue hypoperfusion/hypoxia' and 'mitochondrial dysfunction' theories. Each of these will be discussed briefly.

Inflammation

Given the finding of profound, systemic inflammatory activation in critical illness, it seems likely that the secondary effects are at least partly related to this

phenomenon. Pulmonary failure, in the form of acute lung injury (ALI) or ARDS, is certainly characterised by an influx of inflammatory cells, mostly neutrophils, coupled with loss of alveolar barrier function and non-cardiogenic oedema (Segel, Halterman & Lichtman 2010). Indeed, tissue damage by neutrophil-derived enzymes such as HNE seems to be a major pathophysiological feature of ARDS/ALI (Wang et al. 2009), although treatments aimed at neutralising these in humans have so far proved disappointing (Iwata et al. 2010). During sepsis neutrophils are also found to infiltrate some other tissues, particularly the kidneys (Kabay et al. 2007), gut (Taner et al. 2001) and liver (Shih et al. 2008), and through attachment to endothelium in the microcirculation alter blood flow and increase the risk of clotting and microvascular obstruction (Ferri et al. 2009). Removing neutrophils can reduce or prevent end-organ damage (Poggetti et al. 1992), as can the elimination of circulating monocytes (Kreisel et al. 2010). However it should be noted that ARDS has been found in neutropaenic patients (Heyll et al. 1991), and that the degree of neutrophilic infiltration does not always correlate well with severity of organ damage (Raj, Hazinski & Bland 1985). Immune damage may not result solely from cellular actors. The anaphylotoxins C3a and C5a can produce systemic vasodilatation and increased capillary pore size, so mimicking the redistributive shock which commonly accompanies sepsis and severe SIRS (Lundberg, Marceau & Hugli 1987). C5a has also been implicated in septic cardiac failure (Niederbichler et al. 2006), pituitary failure (Flierl et al. 2009) and dysregulated coagulation pathways (Laudes et al. 2002b).

Monocytes and their resident tissue derivatives, macrophages, play a key role in the immune defence against microbial invasion (Cote et al. 2004, Osterloh et al. 2007). Macrophages are key mediators of the response to PAMPs and DAMPs (Osterloh et al. 2007), and play an important early role in triggering systemic inflammation (reviewed in Laudanski, Wyczechowska 2005), as well as acting as antigen presenting cells to stimulate the adaptive arm (reviewed in Martinez-Pomares, Gordon 2007). Monocytes are also intimately involved in marshalling neutrophil recruitment to sites of inflammation (Kreisel et al. 2010). However there is considerable evidence of monocytes and macrophages expressing an anti-inflammatory phenotype (Newton et al. 2004, Flohe et al. 2008, Mokart et al. 2010), which will be discussed below .

It should not, however, be assumed that sepsis simply involves activation of the innate immune system. Various animal models have implicated T cells in the pathogenesis of organ dysfunction. IL-17, the key mediator of the T_H17 response, has been associated with impaired outcomes in a murine caecal ligation and puncture (CLP) model (Flierl et al. 2008b). T_H17 cells have also been implicated in the formation of intra-abdominal abscesses following intra-peritoneal injection of *Bacillus fragilis* in mice (Chung et al. 2003b). CD8+ve, cytotoxic T cells are also involved in Fas/Fas ligand-mediated liver and splenic cell apoptosis, so contributing to tissue loss and failure in these organs (Wesche-Soldato et al. 2007). Similar findings are noted in sterile insults such as burns (Toth et al. 2004) and ischaemia-reperfusion injury (Miyazawa et al. 2002). Interestingly, although the study of T cells in human sepsis and SIRS remains limited, what data there are suggest that CD4 numbers are depressed (Venet et al.

2010), T cell function reduced (Heidecke et al. 1999) and that there are frequently elevated proportions of immunosuppressive regulatory T cells (T-regs) (Venet et al. 2009), somewhat akin to the situation with monocytes. However when the experimental drug TGN1412 was used to modulate T cells, the resultant dysregulation of the immune system resulted in an intense SIRS, cytokine storm and multi-organ dysfunction (Suntharalingam et al. 2006). The aim of TGN1412 was to expand the T-reg population, so enhancing their immunosuppressive qualities, although the resultant response was the complete opposite of that intended, emphasising the potential risks of modulating the adaptive immune system.

Natural killer (NK) cells are cytotoxic cells whose primary role is thought to be the removal of malignant and infected cells (Farag, Caligiuri 2006). Depletion of NK cells has been associated with reduced inflammation, reduced organ damage and improved survival in murine polytrauma/sepsis models (Barkhausen et al. 2008). Similar to CD8+ve T cells, some of which also display NK type markers (so called NK-T cells), NK cells have also been associated with hepatocyte damage following bacterial sepsis (Seki et al. 2000).

Inflammation, both through proteolytic cascades and activated immune cells, is therefore likely to be a key part of the mechanisms underpinning multi-organ failure following the triggering of acute, uncontrolled inflammation in sepsis and SIRS.

Gastro-intestinal source of organ failure

Animal models of burn injury (Woodruff et al. 1973) demonstrated increased bowel permeability and translocation of bacteria. This, along with the finding of LPS or bacteria in the circulation and lymphatics of patients with critical illness (van Deventer et al. 1988, MacFie et al. 2006), led to the concept of gut hypoperfusion, bacterial translocation and gastro-intestinal immune failure driving systemic inflammation and multiple organ dysfunction (Munster AM, 2000). Interestingly cytokine levels have been found to be grossly elevated in the lymphatic drainage of the gut although this is not always accompanied by endotoxin detection (Deitch et al. 1994). Thus it is possible that factors other than simple translocation of bacteria and bacterial products are driving this inflammatory response. It is now well established that injurious ventilation of the lungs drives an inflammatory response termed 'biotrauma' (to combine volu-trauma, barotrauma and atelectasis as mechanisms of ventilator-induced lung injury (VILI) (Gattinoni et al. 2010), and that this is associated with multi-organ failure. Gut hypoperfusion may well produce a similar immune response (Tamion et al. 2003), so driving systemic inflammation and the mechanisms noted above in immune-mediated organ damage.

Tissue hypoperfusion

Shock has long been recognised as a severe accompanying feature of sepsis and SIRS. Although classically described as a redistributive shock associated with vasodilatation and increased capillary permeability (Parrillo 1993), it is not unusual to find a state of peripheral vascular shutdown, diminished blood volume and impaired cardiac output (Gahhos et al. 1981, Annane, Bellissant & Cavaillon

2005, Maeder et al. 2006). Until recently efforts at resuscitation have been aimed solely at macro-vascular end-points such as mean arterial pressure, cardiac output and global oxygen delivery (da Silva Ramos, Azevedo 2010). However intra-vital microscopy and tissue oxygenation studies in both humans and animals (Sair et al. 1996, Spanos et al. 2010) reveal considerable heterogeneity in micro-vascular blood flow, suggesting complex regulatory failure of micro-vascular homeostasis and resultant tissue hypoxia. As noted above, inflammatory mediators such as C5a (Hugli, Marceau 1985), dysregulated coagulation (Sivula et al. 2009), microthrombus formation (Baskurt, Temiz & Meiselman 1997), and clumping of adherent leukcoytes (Hwang, Han 2003) all contribute to this picture, alongside endothelial failure (Ait-Oufella et al. 2010). Tissue hypoxia can result in cellular death, with the release of alarmins such as HMGB-1 and further exacerbation of the inflammatory cascade (Sims et al. 2010), as well as directly contributing to organ failure. Early therapy aimed at improving tissue perfusion and oxygen delivery have shown considerable benefit in reversing microvascular dysfunction and preventing organ dysfunction (Rivers et al. 2001, Ospina-Tascon et al. 2010, Jhanji et al. 2010), suggesting that this mechanism plays an important role in initiating damage. However 'late' therapy aimed at the same ends does not produce similar results (Ospina-Tascon et al. 2010, Hayes et al. 1994), suggesting that once one is past a certain threshold the processes initiated become irreversible.

Cellular toxicity and mitochondrial deactivation

One of the remarkable features of critical illness-associated organ failure is how often there is little histological evidence of organ damage, such as widespread

necrosis or dense inflammatory infiltrates (Brealey et al. 2004). Although, as ARDS and some forms of acute renal failure demonstrate, this finding is not universal. This has provoked examination of the cellular mechanisms of organ failure (Singer 2007). Indeed survivors of critical illness seldom display chronic organ dysfunction on recovery (Mongardon, Dyson & Singer 2009, Schiffl, Fischer 2008), even in organs that have little regenerative capacity (Knoester et al. 2008). It is also notable that whilst early stage shock demonstrates increased oxygen extraction, as measured by mixed venous oxygen content (C_vO_2), the later stages are characterised by increased C_vO_2 content. This suggests impaired oxygen utilisation, and indeed such findings are associated with poorer outcomes (Shoemaker et al. 1993). It therefore seems that cellular respiration is impaired, and indeed there is growing evidence from both animal and human studies of defects at the mitochondrial level (Brealey et al. 2004, Singer 2007). These may be driven by a range of neuro-humoral mediators from nitric oxide to catecholamines, thyroid hormones to cortico-steroids, levels of all of these being altered in shock states arising from sepsis and SIRS (reviewed in Singer et al. 2004).

Although each of these hypotheses are presented separately, it is highly probable that they operate in an integrated manner, each one capable of driving the others. As a result the patient gets locked into the vicious cycle of immune activation, tissue damage, organ dysfunction and further immune activation that seems to characterise critical illness. The emerging picture is one of complex dysregulation of key body systems, from immunity to coagulation, micro- and macro-vascular functioning to individual solid organs, locking the patient into a

maladaptive state which frequently proves fatal. However what is remarkable, given the central role for immune activation, is the compelling and growing evidence of immune failure, which may, in part, explain the extremely high rate of secondary infection. It is to this topic that we turn next.

Section 1.05 Immune defects in critical illness

(a) SIRS and CARS

As the preceding section has demonstrated, critical illness is characterised by immune activation and organ failure. However, to date, there have been no positive phase III trials of immunosuppressive therapies in sepsis or sterile SIRS and indeed some have shown harm (Fisher et al. 1994, Abraham et al. 1995, Bone et al. 1987). Activated protein C, arguably the only specific therapy for severe sepsis with organ failure, has immunomodulatory effects (Nick et al. 2004) but the extent to which these are responsible for the survival benefit remains controversial (Eichacker, Natanson 2003). It was the failure of anti-inflammatory therapies that prompted Bone to propose the concept of a compensatory anti-inflammatory response syndrome (CARS) in 1996 (Bone 1996). He proposes that the uncontrolled, maladaptive systemic inflammation of SIRS provokes an equally uncontrolled, maladaptive counter-regulatory immunosuppression.

Evidence of immunosuppression in sepsis and SIRS is found in both numbers and functions of immune cells as well as anti-inflammatory soluble mediators. The following section will discuss each of the cell types in turn, followed by a

discussion of possible mediators and mechanisms. Finally we will turn to the time course, and its relationship to episodes of hyper-inflammation.

(b) Monocyte/macrophage hypoactivity

The hypoactivity of peripheral blood monocytes is one of the best-characterised aspects of sepsis-related immune suppression. Patients with sepsis and other critical illnesses including head injury, pancreatitis and fulminant hepatic failure, have monocytes that produce low levels of pro-inflammatory cytokines, such as TNF-alpha. When stimulated *ex-vivo* with ligands including LPS (Wolk et al. 2000, Asadullah et al. 1995, Ho et al. 2006, Berry et al. 2011) they enter a state termed 'monocyte deactivation' (Volk et al. 1996). This state is accompanied by diminished HLA-DR expression (Höflich et al. 2002), so impairing antigen presentation and stimulation of the adaptive arm of the immune system. Low monocyte HLA-DR has been associated with increased risk of nosocomial infection (Landelle et al. 2010) and mortality (Mebazaa et al. 2004). Animals subjected to caecal ligation and puncture (CLP) also display hyporesponsive alveolar and peritoneal macrophages (Chung et al. 2003a, Attalah et al. 2002), and impaired clearance of organisms such as *Pseudomonas aeruginosa* when administered as a 'second hit' (Muenzer et al. 2006). Apoptosis is generally reported to be enhanced amongst monocyte/macrophage populations in sepsis (reviewed in Wesche et al. 2005).

What mediates monocyte deactivation remains disputed, although IL-10 is almost certainly a pivotal player (Sfeir et al. 2001b). However IL-10 is not

sufficient, as levels similar to those found in sepsis are found in other patients such as patients with severe psoriasis (Deeva et al. 2010). These patients do not display monocyte deactivation, and indeed there is some evidence that deactivation can occur in the absence of elevated serum levels of IL-10 (Volk 2003). It therefore seems likely that other factors such as transforming growth factor beta (TGF-beta) (Czarniecki et al. 1988), cortisol (Le Tulzo et al. 2004) and/or catecholamines (van der Poll et al. 1996) may also be involved in mediating this response.

(c) Neutrophils

Neutrophils in patients with sepsis tend to display surface markers of 'activation', notably elevated levels of CD11b and CD64 and diminished CD16 (Berger et al. 1984, Muller Kobold et al. 2000, Eksioglu-Demiralp et al. 2001). However some studies have also demonstrated diminished inducibility of these receptors (Rosenbloom et al. 1999), indicating the potential for impaired activation in severe infections. Despite their accumulation and damage of organs (see above), neutrophils from septic patients display impaired transmigration (McGill et al. 1996) and chemotaxis (Arraes et al. 2006). Other key anti-bacterial functions, such as ROS production and phagocytosis, show a more variable picture, with studies showing both increases (Martins et al. 2003) and decreases (Kaufmann et al. 2006) in these functions. As with monocytes, dysfunctional neutrophils are associated with secondary infections (Stephan et al. 2002) and sepsis-related mortality (Danikas et al. 2008). However, in contrast to monocyte inflammatory cytokine release, septic patients demonstrate enhanced release of proteolytic enzymes such as NE in response to *ex-vivo* LPS (Ertel et al.

1994), as well as showing higher levels in plasma (Nuijens et al. 1992). Similar findings are made when examining other neutrophil granule contents such as myeloperoxidase (Kothari et al. 2010). In contrast to monocytes, neutrophils in patients with sepsis have diminished apoptosis (Taneja et al. 2004), which may lead to persistence and hence worsening organ damage (Paunel-Gorgulu et al. 2011).

Several mediators have been proposed as key to these effects. Animal models and *in-vitro* work have identified an apparently paradoxical effect of the inflammatory molecule C5a in impairing phagocytosis and the generation of ROS (Huber-Lang et al. 2002b) as well as inhibiting apoptosis (Guo et al. 2006) and stimulating degranulation (Gardinali et al. 1992). These effects have also been shown in non-septic models such as trauma (Flierl et al. 2008a, Amara et al. 2010b). In decompensated alcoholic liver disease, a condition that bears some comparison with sepsis (Sen, Williams & Jalan 2002), circulating LPS and ammonia have been linked to defects in phagocytosis (Mookerjee et al. 2007, Shawcross et al. 2008). Impairment of IL-17 signalling has been linked, in knock-out animal models, to impaired neutrophil migration (Cho et al. 2010), although the relevance of this to human SIRS (which typically reveals elevated levels of IL-17 (Finnerty et al. 2008)) remains uncertain. Elevated levels of IL-10 have also been associated with impaired neutrophil recruitment (Sun et al. 2009), although whether this is a direct effect or mediated by impairing other cellular actors is uncertain.

(d) Dendritic cells

Alongside macrophages, dendritic cells (DCs) play a key role in antigen presentation and activation of the adaptive immune system (Steinman 1991). Human *post-mortem* studies reveal depletion of lymph node DCs in sepsis (Hotchkiss et al. 2002), whilst animal models of sepsis confirm this finding and demonstrate similar depletion of splenic and pulmonary DCs (Efron et al. 2004). In addition animal models show DCs skewed towards an anti-inflammatory phenotype, releasing IL-10 and suppressing the release of IL-12 in response to LPS (Karp et al. 1998, Yanagawa, Onoe 2007). The mediators of depletion remain uncertain, although interestingly the PAMP receptors TLR2 and 4 are involved in the process, mediating apoptosis of DCs (Pène et al. 2009).

(e) T lymphocytes

T lymphocytes can be divided into cytotoxic (CD8 positive) and helper (CD4 positive, T_H) subsets, with the former mediating lysis of infected and malignant cells, whilst the latter act to stimulate adaptive immune responses following exposure to specific antigens. T cells also have important interactions with innate immune cells, such as monocytes, macrophages and neutrophils (Pelletier, Micheletti & Cassatella 2010, Walz et al. 2009, Tiemessen et al. 2007). As noted above, T_H cells come in several different sub-types, each of which produces distinct groups of cytokines, which invoke distinct responses. T_H1 cells produce cytokines including interferon gamma (IFN-γ), and TNF-beta stimulating a predominantly cytotoxic response. In contrast T_H 2 cells produce IL-4, IL-5 and IL-10 which stimulate more B-cell/humoral mediated responses (Mosmann, Coffman 1989) whilst also dampening down functions of other immune cells

such as monocytes (Yoshida et al. 2001). T_H 17 cells, a more recently identified subset, produce IL-17 and IL-6, and appear to be involved in the clearance of bacterial and fungal pathogens (Cho et al. 2010). Given this role, it is unsurprising that IL-17 receptor deficiency is associated with poor outcomes in sepsis (Freitas et al. 2009), although as noted above systemic release of IL-17 is associated with organ failure and mortality (Flierl et al. 2008b).

A further subset of T_H cells is the regulatory T cells (T-regs), which are thought to arise from several lineages (reviewed in Langier, Sade & Kivity 2010). These cells have immunosuppressive effects, limiting T cell proliferation in response to antigen, secreting IL-10, switching macrophages to an M2 phenotype and impairing neutrophil functions (Venet et al. 2009, Tiemessen et al. 2007, Suri-Payer, Cantor 2001, Lewkowicz et al. 2006). Most T-regs arise from the thymus, and unusually appear to be generated by recognition of self-MHC molecules, a response which would normally result in clonal deletion (reviewed in Langier, Sade & Kivity 2010). These natural T-regs (nT-regs) are supplemented by the generation of regulatory phenotype in the periphery by CD4+ T-cells exposed to high levels of TGF-β, so called induced T-regs (iTregs) (Shevach et al. 2008). Both n and i T-regs are characterised by the expression of Forkhead box P3 (FOXP3), a cytoplasmic transcription factor that is crucial to T-reg function (reviewed in Langier, Sade & Kivity 2010). Deletion of FOXP3 results in multi-system autoimmune disease in mice (Brunkow et al. 2001) and, in humans, the fatal immune disorder immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) (Bennett et al. 2001). Most of the work on T-regs to date has focused on their role in auto-immunity, with several auto-immune

diseases showing depressed levels of these cells including rheumatoid arthritis, diabetes, multiple sclerosis and systemic lupus erythematosus (reviewed in Langier, Sade & Kivity 2010).

Sepsis induces T cell anergy (Heidecke et al. 1999), although until recently the mechanisms underlying this remained obscure. However recent work has indicated a role for elevated levels of T-regs in this process (Venet et al. 2009). Indeed several groups have now identified elevated levels of T-regs in patients and animals with sepsis (Venet et al. 2009) and following major trauma (Venet et al. 2008). Lymphocytes also reportedly undergo enhanced apoptosis in sepsis, potentially contributing to impaired immune responses (Schwulst et al. 2006).

As with the other cell types discussed above, it appears likely that multiple mechanisms are involved in the impairment of T cell functioning. As examples, T cell apoptosis is enhanced by C5a (Guo et al. 2000), as well as by ligation of the peroxisome proliferator-activated receptor γ (PPARγ) (Soller 2005) and endogenous corticosteroids (Ayala et al. 1999) all of which are relevant to sepsis. As noted above T-reg levels are elevated in some patients with sepsis (Venet et al. 2009) and major injury (Venet et al. 2008), and impair proliferative responses of both CD4 and CD8 positive cells (Venet et al. 2009). Polarisation of T-cells towards a regulatory phenotype is induced by cytokines including IL-6, IL-10 and TGF-beta (Shevach et al. 2008), levels of which are commonly elevated in sepsis and other forms of critical illness (Leonidou et al. 2007).

Although presented by cell type above, it is likely that these defects and dysfunctions co-exist although to date few studies have attempted to examine the function of more than one immune cell at any one time. Furthermore it is likely that simultaneous immune defects are not simply the result of common mediators, but also the interactions between dysfunctional cells resulting in complex synergies, which amplify and enhance existing defects.

(f) Time course of immunosuppression and hyperinflammation

One possible reason for the variable and apparently contradictory findings of immune cell function from different reports may be the time point at which the measurement is made. In animal models, where pre-morbid sampling is feasible and there is certainty over time and severity of onset, most studies indicate a rapid pro-inflammatory response with immune cell activation/hyperactivity with later onset of hypoactivity (Hotchkiss, Karl 2003). In human studies, monocyte deactivation is largely reported to occur several days after admission to intensive care (Meisel et al. 2009), as are T cell impairments (Venet et al. 2009). However this is not universally the case and apparently 'early onset' immune suppression is noted in some reports (Danikas et al. 2008, Lukaszewicz et al. 2009). In these latter cases it is possible that the early 'hyperactive' phase may well have been missed in the pre-ICU period. What is also clear is that the 'immune status' (i.e. hyper- or hypo-active) may depend on which aspect is examined, with increasing evidence that both pro- and anti-inflammatory mediators may be elevated at the same time (Osuchowski et al. 2006), leading to the coining of the terms 'mixed anti-inflammatory response syndrome' (MARS) (Bone 1996).

Immunophenotyping of cellular functions has lagged some distance behind

measurement of serum cytokines and adds further complexity to any assessment of 'immune system functioning'. Questions remain over whether hyper-inflammation can be recrudesced following second or third 'hits' (such as nosocomial infections), and indeed whether immune cells such as neutrophils may be able to be simultaneously tissue-toxic but unable to combat microbial invasion?

Section 1.06 The immune system in critical illness- a summary

The section 1.04 above describes an emerging picture of critical illness, triggered by an initial immune-activating response which, if not rapidly treated and terminated, leads on to immune-mediated organ damage and a downward spiral of maladaptive processes which further exacerbate the immune stimulation, as well as directly and indirectly damaging organ systems. The immune system itself is not protected from this damage, and can also in turn suffer impaired functioning leading to inability to deal with microbial invaders and so facilitating the nosocomial infections discussed earlier in this chapter.

Section 1.07 Physiological functioning of neutrophils in clearance of microorganisms

(a) Neutrophil priming and activation

We now turn to the physiological functioning of one particular immune cell, the neutrophil, to discuss its anti-microbial functions in health in contrast to the pathological situations described above.

The neutrophil is the most abundant blood leukocyte, existing in a circulating pool and a smaller number of marginated cells ready to enter extra-vascular tissues should they be required (Kuebler 2005, Summers et al. 2010). On stimulation by factors such as C5a (Kubo et al. 1998), leukotriene B4 (LTB4) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (reviewed in Jagels, Hugli 1994), the bone marrow pool releases neutrophils into the circulation. Myelopoeisis is stimulated, leading to the neutrophilia, which is common to a variety of septic and sterile insults. Acquired or congenital absence of neutrophils leads to enhanced susceptibility to a variety of infections, mostly bacterial and fungal (Julia et al. 1991, Shiohara et al. 2009).

On exposure to molecules such as formylated peptides, C5a, IL-8 or platelet activating factor (PAF), neutrophils adopt a 'primed' form (Condliffe et al. 1996), with enhanced expression of adhesion molecules, phagocytic receptors and readiness to produce ROS. A second 'hit' by another activating molecule can then provoke release of ROS or proteolytic enzymes such as HNE (Condliffe et al. 1996, Bhatia et al. 2006). These 'activating' molecules can also act as chemoattractants, allowing cells to move towards targets (reviewed in Walker, Ward 1992). Neutrophils need to exit the circulation to enter tissues to combat microbial invaders or remove dead tissue. This process requires adhesion to the endothelium, rolling and then transmigration, again moving down chemotactic gradients (reviewed in Borregaard 2010). Various in-born errors of neutrophil metabolism have been identified, including leukocyte adhesion deficiency (LAD), where the adhesion molecule CD18 is absent or non-functional and neutrophils experience impaired adhesion and transmigration resulting in

increased susceptibility to infection (Kuijpers et al. 1997, Hayward et al. 1979). Defects in the NADPH oxidase machinery required for the production of ROS, which results in chronic granulomatous disease (CGD) (Winkelstein et al. 2000), whilst defects in beta-actin polymerisation are associated with impaired chemotaxis, phagocytosis and recurrent infections (Nunoi et al. 1999).

(b) Mechanisms of phagocytosis

Phagocytosis, the process by which cells engulf or ingest particulate matter, was first described in mammalian cells by Metchnikoff in 1883 (Metchnikoff, 1883). Although often referred to as a single process, it is in reality a collection of different processes, each of which operate via differing receptors, differing signalling pathways and with differing cellular responses. For the purposes of this discussion, the focus will be on neutrophil phagocytosis of micro-organisms, and most specifically that mediated by complement opsonisation.

Although neutrophils can recognise and phagocytose invading micro-organisms by non-specific scavenger receptors, phagocytosis is far more efficient when the target has been coated in an 'opsoniser' (Blom, Hallstrom & Riesbeck 2009, Palecanda, Kobzik 2001, Nimmerjahn, Ravetch 2006). Two main classes of opsonin exist, immuno-globulin and especially the gamma sub-class (IgG), and complement component C3b. IgG production, as with all antibody responses in the mature animal, relies on exposure of B-cells to the triggering molecular pattern alongside co-stimulation signals from the presenting cell. However circulating antibody levels and rapid mobilisation of memory B-cells can result

in a rapid response if the target has been encountered before (Nagafuchi 2010). Complement is discussed below (section 1.08), although it is worth noting that anti-bodies bound to pathogens can activate complement, providing further links between the innate and adaptive immune responses.

Phagocytosis is initiated by the appropriate binding of a target to a phagocytic receptor. The predominant receptors are the Fcγ receptors for IgG, which bind to the common 'Fc' portion where the 'specific' portion has bound to the phagocytic target, and the complement receptors, specifically CR3 (also known as Mac-1 or CD11b/CD18). Binding to a phagocytic receptor triggers clustering of further phagocytic receptors, intracellular signalling and resultant actin polymerisation and cellular morphology change to permit phagocytosis (Lee, Harrison & Grinstein 2003). There is an apparent morphological divergence in mechanisms of phagocytosis, with Fcγ activation producing a pseudopodal engulfment whilst CR3 activation results in the target 'sinking' into the cell (Kaplan 1977). Fcγ receptors are activated as soon as a target binds to them. In contrast CR3 requires prior activation (e.g. by C5a, LPS or similar) (Jones et al. 1998, Fallman, Andersson & Andersson 1993) leading to conformational change and exposure of the activation epitope (Diamond et al. 1993) before binding will activate phagocytosis.

Binding of ligands to CR3 induces tyrosine kinase activity, specifically syk, which in turn activates protein kinase C, initiating RhoA activation, actin polymerisation and phagocytic ingestion (Caron, Hall 1998, Shi et al. 2006b). In contrast Fcγ activates both syk and Lyn tyrosine kinases (Strzelecka-Kiliszek,

Kwiatkowska & Sobota 2002), resulting in a variety of intracellular pathways including phospholipase C, PI3K and phospholipase A2 (Garcia-Garcia, Rosales 2002)) and then Rac-1 and CDC42 ,which mediate the distinct actin polymerisation and pseudopodal formation noted above (Kaplan 1977, Caron, Hall 1998).

Following ingestion the cell forms a 'phagosome', an intracellular inclusion containing the target. This is fused with intracellular granules containing proteolytic enzymes such as NE, and ROS generators such as myeloperoxidase and NADPH oxidase complex translocation, which then kill the organism ingested in the 'mature' phagolysosome (Lee, Harrison & Grinstein 2003). There is also extracellular release of proteolytic enzymes and ROS, aimed at killing organisms that have not been ingested (Ohlsson, Olsson 1977). It is this extracellular release that is thought to lead to tissue damage, such as that seen in critical illness (Matsuda, Hattori 2006, Yao et al. 1998). Disruption of any of the above processes, from ligation of phagocytic receptors through actin polymerisation and maturation of the phagolysosome can impair phagocytosis and/or intra-cellular killing.

Section 1.08 Complement function in inflammation and infection

The complement system consists of a collection of plasma proteins, predominantly manufactured in the liver, which ordinarily exist in an inactivated form (zymogens). On activation there is a cascade of proteolysis, each preceding molecule activating the next in the chain in a manner analogous to the coagulation cascade (Walport 2001a, Walport 2001b). The final endpoint of the

complement cascade is the membrane attack complex (MAC), which is formed by components C5-C9, and which acts to punch holes in membranes, so producing cellular lysis. However along the pathway a number of other products are produced, which play key roles in anti-bacterial defence.

The complement system can be triggered by one of three pathways, namely the 'classical', 'alternative' and 'mannose binding lectin' (MBL) pathways (Walport 2001a). Each of these are triggered by different stimuli, the classical pathway by IgG antibody bound to an antigen or immune complex, the alternative pathway by LPS and the MBL pathway by lectins bound to mannose on bacterial cell walls. These three pathways then converge on C3. C3 is continually being activated even without up-stream stimulation, as the enzyme is subject to hydrolysis (Pangburn, Muller-Eberhard 1983), although this happens at a low level. On activation C3 splits into 2 products, C3b and the smaller C3a. C3b acts as an opsonising agent, facilitating phagocytosis via the CD11b/CD18 integrin (see 1.07 b above). Once C3b is deposited on a cell wall it can bind to factor D, resulting in autocatalytic deposition of further C3b - facilitating phagocytosis - and triggering of MAC formation (Forneris et al. 2010, Fearon, Austen & Ruddy 1973). To prevent C3b undergoing this process on healthy host cells several inhibitors exist. These include factors H and I that bind and inactivate C3b (Brown et al. 1983), as well as decay accelerating factor (DAF/CD55) and CD59 which are cell surface inactivators of C3b (Harris et al. 2007, Brooimans et al. 1992). C3a is an anaphylatoxin, which although less potent than C5a (Ehrengruber, Geiser & Deranleau 1994), is none the less capable of producing vasodilatation, vascular leak and hypotension. It is also

chemotactic for eosinophils, although only indirectly so for neutrophils (Hugli, Vallota & Muller-Eberhard 1975, Daffern et al. 1995). Activated C3 forms C5 convertase, which splits inactive C5 into two products, C5b and C5a. C5b forms the first part of the MAC, whilst C5a is the more potent anaphylatoxin producing similar responses to C3a (Hugli, Marceau 1985, Lundberg, Marceau & Hugli 1987).

C5a has a very short plasma half-life (2-3 mins) (Webster, Larsen & Henson 1982), and is removed by binding to its predominant receptor CD88 (Chenoweth, Hugli 1978). Bound C5a is internalised by CD88-bearing cells such as neutrophils, where it is metabolised prior to CD88 being recycled to the cell surface (Oppermann, Götze 1994). Plasma carboxypeptidases cleave the terminal arginine from C5a to form the 'des-arg' product (Campbell et al. 2002), which is less potent than intact C5a (Webster et al. 1980) and is cleared by binding to CD88 (Webster et al. 1980, Marder et al. 1985). There is also some non-neutrophil contribution to plasma C5a clearance from highly perfused organs including the lung, liver and kidneys (Webster, Larsen & Henson 1982).

As well as activation at the start of the cascade, several non-complement proteins can lyse C5 into its split products. These include thrombin, from the coagulation cascade (Amara et al. 2010a), and NE released by activated neutrophils (Wetsel, Kolb 1983). Both of these can themselves be activated/triggered by activated complement (Kambas et al. 2008, Binder et al. 1999). Although the majority of C5a is generated by the complement cascade described above, macrophages and neutrophils can also generate it from C5 directly (Huber-Lang et al. 2002a). These non-complement-mediated activators of C5 are not constrained by the

factors that inhibit C3 (see above) and therefore may provoke less controlled complement release. This illustrates how disparate inflammatory mechanisms triggered during sepsis and SIRS can amplify each other and so drive the maladaptive processes seen in critical illness.

CD88 is a hepta-helical, transmembrane receptor that is coupled to G-proteins, the G_{α} subunit being $G_{\alpha i2}$ (Chenoweth, Hugli 1978, Skokowa et al. 2005). On C5a binding to CD88 a number of intra-cellular pathways are triggered, including the adenylate cyclase/cyclic AMP/protein kinase A pathway, PI3K pathway, extracellular signal-regulated kinase 1/2 (ERK1/2) and protein kinase C (PKC) pathways (Tsu, Allen & Wong 1995, Riedemann et al. 2004, Wrann et al. 2007b). C5a binding also results in receptor phosphorylation and desensitisation (Christophe et al. 2000), as well as receptor sequestration and consequent diminution of cell-surface expression (Bock et al. 1997, Braun 2003). Interestingly these processes (desensitisation and sequestration) appear to be mediated by divergent pathways (Christophe et al. 2000). There is a second C5a receptor, termed C5L2, which is not coupled to G-proteins. As a result this was long thought to be a 'decoy' receptor, which was present simply to mop-up C5a and reduce CD88 activation. However recent work using knock-out models and receptor inhibitors have indicated a functional role for C5L2 in the pathogensis of several diseases including sepsis induced multi-organ failure (Rittirsch et al. 2008) and allergic asthma (Zhang et al. 2010b). Interestingly the greater proportion of C5L2 appears to be contained in intra-cellular granules, with the suggestion that it exerts its effect on C5a transported intra-cellularly by CD88 (Bamberg et al. 2010), although what the precise effects mediated by C5L2 on intra-cellular pathways are remains uncertain (Ward 2009).

Section 1.09 Chapter summary and hypotheses

Critical illness presents the clinician with something of a paradox. It is characterised by systemic inflammation, resulting in organ failure, in which activated immune cells and proteolytic cascades are highly implicated. However, these patients are also highly susceptible to nosocomial infections, and whilst various clinical factors have been proposed to explain this susceptibility, none appear to adequately deal with the underlying host susceptibility.

It is notable that despite clinical (e.g. pyrexia, hyperaemia and extravasation of plasma) and laboratory (e.g. leukocytosis, hypercytokinaemia) evidence of inflammation during critical illness, those studies which have examined immune cellular *function* in patients are more likely to show hypofunction, 'deactivation' and anergy rather than hyperactivity. Indeed it appears that alongside other forms of organ failure, we can add 'immune failure', to the burden of critical illness.

These features produce several hypotheses that will be addressed by this thesis.

1) Critically ill patients exist in a state of simultaneous hyper-inflammation and immune failure.
2) Human neutrophil dysfunction in critical illness is driven by excessive pro-inflammatory stimulation by C5a.
3) C5a-mediated neutrophil dysfunction is associated with an increased risk of nosocomial infection.

4) C5a-mediated neutrophil dysfunction co-exists with other forms of immune hypoactivity, and these factors interact to influence the risk of nosocomial infection.

Section 1.10 Thesis overview

Chapter 2 will deal with the experimental methods used for this study. Chapter 3 will present the first results, examining the first hypothesis in patients with clinically suspected ventilator-associated pneumonia. Chapters 4 and 5 will address the second hypothesis, examining the role of C5a in mediating dysfunction in patients and exploring the underlying mechanism by which C5a inhibits the key function, phagocytosis. The final results chapter, Chapter 6, will address the final two hypotheses by exploring the relationship between immune dysfunction and the acquisition of nosocomial infection. The discussion of results and conclusions will form the final chapter.

Article II. Materials and Methods

Section 2.01 *Introduction and chapter overview*

This chapter will present the scientific and statistical methods used in the preparation of the thesis. For a variety of reasons some of the techniques used differed between different parts of the study (specifically the blood collection, flow cytometry and neutrophil extraction protocols) and so sections of the results to which the specific method pertains will be detailed, however the methods will be ordered by technique and application rather than by the order of the thesis.

Four groups of human subjects were involved in this work, one group of critically ill patients with clinically suspected VAP (VAP cohort), one group of volunteers who were age- and sex-matched to the VAP cohort (matched volunteers), a further group of critically ill patients recruited prior to developing nosocomial infection (prospective cohort) and finally a group of healthy, young (<50) university employees (healthy volunteers). Each of these groups will be reported in more detail below.

Section 2.02 Materials and reagents

The A549 alveolar epithelial cell line was obtained from ATCC (Teddington, UK).

Anti-human CD88/Alexa647 was obtained from AbD Serotec (Kidlington,UK).

The following materials and reagents were acquired from Abcam (Cambridge, UK): murine monoclonal anti-human CD11b antibody M1/70, murine monoclonal anti-human CD16 antibody 3G8, murine monoclonal anti-human CD88 antibody S5/1, murine monoclonal anti-human antibody CD88 P12/1.

Recombinant human granulocyte-macrophage colony stimulating factor (GM-CSF) was from Affinity Bioreagents (Golden, CO, USA).

Polymorphoprep was acquired from Axis-Shield (Dundee, UK).

The following materials and reagents were acquired from Becton Dickinson (BD Bioscience, Oxford, UK): Facslyse™, Futhan, murine Quantibrite™ anti-human HLA-DR/monocyte antibody, and Quantibrite™ calibration beads. C3a-des arg, C5a-des arg, GM-CSF, G-CSF, IL-1β, IL-6, IL-8, IL-10, MCP-1, MIP-1α ,TNF-α and soluble CD62L cytometric bead array (CBA) tests were also acquired from BD, as well as ELISAs for C3a-des arg and C5a-des arg.

Quantichrom™ colourmetric urea assay was obtained from BioAssay Systems (Hayward, CA, USA).

Platelet activitating factor (PAF) was obtained from Calbiochem (Nottingham, UK).

The following materials and reagents were acquired from Corning (Lowell, MA, USA): tissue culture plates, and transwell inserts of 3μM and 0.5μM pore size.

The following materials and reagents were acquired from Cytoskeleton (Denver, CO, USA). RhoA, CDC42 and Rac 1,2,3 G-Lisas™, cell lysis solution, Precision Red protein estimation solution.

The following materials and reagents were acquired from Ebioscience (San Diego, CA, USA): murine antibody against the activation epitope of CD11b, CBRM1/5 labelled with fluorescein-5-isothiocyanate (FITC), murine anti-human CD4/FITC and CD25/Allophycocyanin (APC), murine anti-human FOXP3/R-Phycoeyrthrin (R-PE) and R-PE conjugated isotype control, red cell lysis buffer, flow staining buffer and cell fixation/permeabilisation solution.

The following materials and reagents were acquired from Hycult biotechnology (Uden, NL): neutrophil elastase and myeloperoxidase ELISAs.

The following materials and reagents were acquired from Invitrogen (Paisley, UK): Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), FITC-labelled phalloidin, Iscove's modified Dulbecco's medium (IMDM), L-glutamine, murine IgG1 isotype control, polyvinylidene fluoride (PVDF) membrane, murine monocloncal anti-human-CD11b/PE), -CD11b/FITC, -CD16 (Fcγ receptor IIII) /Tricolour (TC), -CD32 (Fcγ receptor II)/FITC, -CD62L (L-selectin)/TC and -CD64 (Fcγ receptor I)/R-PE, as well as similarly labelled murine isotype controls, and goat-anti mouse antibody/alexa-647.

Reastain Diff-quik was acquired from Reagena (Toivala, Finland).

The following materials and reagents were acquired from R&D systems (Minneapolis, MN, USA): cyclic adenosine monophosphate (cAMP) Parameter™ detection kit, polycloncal rabbit anti-human phospho-AKT (S473) antibody, horseradish peroxidase-conjugated goat anti-rabbit antibody.

The following materials and reagents were acquired from Sigma-Aldrich (Gillingham, UK): recombinant human C5a (rhC5a), calcium chloride, cytochrome C, dextran, ethylenediaminetetraacetic acid (EDTA), formalin, lipopolysaccharide (LPS) O127:B8, Luria-Bertani (LB) broth and agar, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), heparin sodium, isoproterenol hydrochloride, Percoll, phosphate buffered saline (PBS), sodium citrate, Triton, Tween 20, Tris-buffered saline, wortmannin, and zymosan.

The following small molecule pathway modulators were acquired from Tocris Bioscience (Bristol, UK): AS605240, IC87114, LY294002, SQ22536, and 740 Y-P.

The following materials and reagents were acquired from Thermo Scientific (Loughborough, UK): bicinchoninic acid (BCA) protein assay, HALT™ protease and phosphatase inhibitor with EDTA, M-PER™ cell lysis buffer, Supersignal™ chemiluminescent detection reagent, and 12% Pierce Precise protein gel.

Section 2.03 Patient and volunteer recruitment

(a) Settings

Patients were recruited from two university hospital general ICUs. Unit 1 (Royal Infirmary of Edinburgh, RIE) is an 18-bedded teaching hospital medical-surgical ICU which admits approximately 1000 patients per annum from all specialties with the exception of cardiac surgery and isolated neuro-trauma. Around 50% of patients admitted to this unit stay for ≥48 hours (i.e. are at increased risk of ICU-AI), and 75% require level 3 care (i.e. invasive ventilation and/or support of two or more organ systems) (UK Department of Health, 2000). Unit 2 (Western General Hospital (WGH), Edinburgh) is a mixed ICU/high dependency unit (HDU) with 16 beds, admitting approximately 800 patients per annum from a mixture of medical, surgical and neurosurgical specialties. 60% require level 3 care, and approximately 71% stay for more than 48 hours.

(b) Patients with suspected ventilator-associated pneumonia (VAP cohort)

(Chapters 3 and 4)

This prospective, observational, cohort study was performed in both unit 1 and unit 2. Patients were screened daily for possible VAP, and were assessed for enrolment as soon as the clinical diagnosis of VAP was made. Where enrolment occurred bronchoscopy took place within 4 hours of clinical diagnosis. All patients had demographic details recorded, including co-morbidities and prescribed medications. The severity of presenting illness was assessed by an acute physiology and chronic health evaluation II (APACHE II) score calculated within 24 hours of ICU admission. Patients were eligible if they fulfilled

recognised criteria for *clinically suspected VAP* i.e. mechanical ventilation for at least 48 hours, new and persisting infiltrates on a chest radiograph and at least two of the following – purulent tracheal secretions, temperature >38°C, or white cell count $>11x10^9$ per litre, criteria based on a modification of previously published clinical criteria (Chastre, Fagon 2002). Exclusion criteria comprised PaO_2<8kPa on FiO_2>0·7, positive end-expiratory pressure >15cmH_2O, active bronchospasm, myocardial infarction within the last 3 months, unstable arrhythmia, mean arterial pressure <65mmHg on vasopressor therapy, bleeding diathesis (including platelet count $<20x10^9$ per litre) and initiation or modification of antibiotics in the preceding 3 days (Chastre, Fagon 2002). Patients who had been on unchanged antibiotics for more than three days were included. Of 74 eligible patients, 73 were enrolled with one excluded due to lack of a relative's informed assent.

Eligible patients had fibreoptic bronchoscopy and BAL performed by a single experienced operator using a pre-defined, standardised technique (Meduri, Johanson 1992, Baselski, Wunderink 1994). Briefly, where focal infiltrates were present, the bronchoscope was wedged in a subsegment corresponding to the area of radiological involvement. In the case of diffuse radiographic change the bronchoscope was wedged in a subsegment producing visible purulent secretions or (in the absence of purulent secretions) in the posterior segment of the right lower lobe. Twenty ml of sterile saline were instilled and the aspirate (representing a 'bronchiolar' sample) discarded, then 200ml of sterile saline was instilled in aliquots and the aspirate (representing an alveolar sample) retained. Whole blood was collected into 0·38% sodium citrate (final concentration).

(c) Age- and Sex-matched volunteers

(Chapters 3 and 4)

After recruitment of 40 patients the (anonymised) age and sex of each patient was communicated to a local primary care practice, where staff unconnected with the study randomly identified matching individuals and sent out invitations to participate. The first twenty-one respondents were enrolled to form a reference group. Exclusion criteria comprised hypoxia (SaO_2 <92% on air), bleeding diathesis, anticoagulant therapy, insulin-dependent diabetes mellitus, arrhythmia, bronchospasm not responding to nebulised β2 agonist, or clinical evidence of respiratory tract infection. Eligible volunteers provided blood and had fibreoptic bronchoscopy performed by the same investigator as above.

(d) Patients admitted prior to any acquisition of nosocomial infection (Prospective cohort)

(Chapters 5 and 6)

This cohort was recruited solely from unit 1. Critically ill patients, defined as those admitted to ICU and requiring exogenous support of one or more organ systems (invasive ventilation, inotropes/vasopressors or haemofiltration) and predicted to require such support for 48 hours or more, were screened for recruitment. Exclusion criteria were: age <16; pregnancy; known human immunodeficiency virus (HIV) infection; known in-born errors of neutrophil metabolism; haematological malignancy; use of immunosuppressive drugs other than corticosteroids; and those thought unlikely to survive for more than 24 hours. Informed consent was obtained directly from patients where possible, otherwise informed assent was obtained from the next of kin. Whole blood was

collected into heparin or EDTA/Futhan. Subsequent EDTA/Futhan samples were taken at 48 hours, then every 2 days until day 7 and then every 3-4 days until a study end-point was achieved. Study end-points were ICU-acquired infection (see below for definition), death without ICU-acquired infection or discharge from ICU without ICU-acquired infection. Definitions of infection are shown below in section 2.03 f, page 76.

(e) Healthy volunteers

(Chapters 4, 5 and 6)

Healthy members of University of Edinburgh staff were identified from a register of research volunteers and asked to provide a sample of blood. For the work presented in Chapter 4, blood was collected as per 2.03 b above (i.e. into sodium citrate at final concentration of 0.38%). For the work presented in Chapters 5 and 6 blood was collected as per 2.03 d above (i.e. into heparin or EDTA/Futhan).

(f) Defining infections for prospective cohort

(Chapter 6)

For patients recruited into the prospective cohort (section 2.03 d above) the outcome of 'infection' was determined by meeting one of the following criteria, based on those provided by HELICS (HELICS 2004).

Any new infection occurring after 48 hours of ICU admission was deemed 'ICU-acquired'. For consistency infections arising within 48 hours of ICU discharge were deemed 'ICU-acquired'.

Infections were defined prior to the start of the study as follows, based on the HELICS criteria.

a) Ventilator-associated pneumonia: Requires radiographic, clinical and microbiological criteria to be met:

i. Radiological criteria.

CXR or CT scan showing new infiltrates, or worsening infiltrates without evidence of pulmonary oedema, and either pyrexia of >38°C or white cells >12000/mm^3 or <4000/ mm^3.

These must be combined with one or more clinical criteria.

ii. Clinical criteria.

- Worsening oxygenation – any increase in FiO_2 to maintain PaO_2 target, or an increase in PEEP, frequency or tidal volume, proning or paralysis to facilitate ventilation.

- Relevant clinical chest findings – auscultatory finding of crepitations, crackles or decreased air entry.

- Increased/changed sputum – any increase in volume, presence of muco-purulent or muco-purulent-bloody sputum.

iii. Microbiological criteria.

The above radiological and clinical criteria must be combined with positive quantitative BAL culture of $>10^4$CFU/ml or positive pleural fluid or pulmonary/pleural abscess culture.

Where the diagnosis of VAP has been suggested by mini-BAL, endotracheal aspirate or where growth is below the 10^4 CFU/ml BAL threshold or without any positive microbiology, adjudication is required.

Hospital-acquired pneumonia (HAP), i.e. nosocomial pneumonia in non-mechanically ventilated patients, requires the same fulfilment of criteria as VAP except that sputum cultures with heavy growth of a single organism constitute a confirmed infection.

b) Catheter-associated infections

Positive culture (semi-quantitative >15CFU) from an indwelling vascular line combined with either

- Local inflammation and pus (catheter-related infection (CRI) or
- Improvement of inflammatory markers within 48 hours of removal (CRI) or

- Culture of the same organism from a peripheral blood culture (catheter-related blood stream infection (CRBSI).

c) Blood stream infection

One positive culture of a typical pathogen, coupled with evidence of systemic inflammation (WCC >12,000/mm^3 or <4000/mm^3, temperature $\geq$ 38°C).

d) Urinary tract infection

Growth of 2 or fewer organisms at $\geq 10^5$ CFU/ml combined with evidence of systemic inflammation (WCC >12,000/mm^3 or <4000/mm^3, temp >38°C or shock without another identifiable cause).

e) Soft-tissue or surgical site infection

Evidence of pus/inflammation at site of presumed infection combined with a positive culture.

Suspected infections that did not meet these criteria were referred to the consensus panel for adjudication. The panel was constituted from a pool of five experienced doctors, 3 intensivists, 1 respiratory physician and 1 microbiologist, all of whom had at least 12 years post-registration experience.

Consensus panel members were asked to come to an independent decision as to whether an infection was 'confirmed', 'probable' or 'unlikely'.

A 'confirmed' infection was where the panel member was convinced that infection was present and would definitely initiate antibiotic treatment and/or pursue source control. Positive microbial detection using techniques other than culture (e.g. by Gram film or PCR), microbial cultures from a normally sterile site or serology confirming a pathogen were obligatory, alongside other clinical evidence of infection.

A 'probable' infection is where the panel member thought there was, on the balance of probabilities, an infection present and would consider antibiotic treatment and/or source control if the patient's clinical condition merited it. This category may include positive microbial cultures. An example would be culture of a classically non-pathological organism (e.g. single cultures of coagulase negative cocci or diphtheroids) associated with clinical evidence of infection/systemic inflammation.

An 'unlikely' infection is where the panel member thought there was a low probability of infection and would not consider antibiotic treatment and/or source control. Although positive microbial cultures could be included in this, this would be culture of a classically non-pathological organism (e.g. single cultures of coagulase negative cocci or diphtheroids) without evidence of systemic inflammation/infection or mixed growth of commensal organisms.

Systemic evidence of infection would require the presence of SIRS – specifically 2 or more of the following: heart rate>90 beats per minute, WCC>12/mm^3 or <4/mm^3 or >10% band types, respiratory rate >20 breaths per minute, or

mechanical ventilation and temperature of >38°C or <36°C. Additional evidence to consider would include reports of large numbers of neutrophils on sample microscopy, and clinical examination findings of pus or inflamed tissue.

'Unlikely' infection combined with a positive microbial culture would constitute colonisation.

The consensus panel was constituted by two members drawn from the pool, and asked to arrive at an independent opinion from one of the three categories above. Where there was agreement the verdict stood, where there was disagreement the panel members met to try and agree a consensus view. If this failed a third member was drawn from the pool and asked to make an opinion between the two options selected by the initial panel members.

(g) Separation and extraction of peripheral blood leucocytes

i) Percoll gradients

(Chapters 3 and 4)

Using a well-established protocol (Haslett et al. 1985), 30 ml of citrated whole blood was separated into cellular component and plasma by centrifugation. Serum was prepared by adding 1M calcium chloride to plasma. Peripheral blood neutrophils (PBNs) and mononuclear cells were isolated from the cellular pellet by sequential dextran sedimentation and Percoll gradient extraction. Purity was

assessed by cytospin and Reastain Diff-quik staining, and cellular number assessed by haemacytometer. Preparations of >95% purity were used for experimental assays and were used fresh (within 30 mins of extraction) in all cases. Cells were suspended at $10x10^6$/ml in warmed IMDM prior to use.

ii) Polymorphoprep

(Chapter 5)

Neutrophils were extracted from heparinised whole blood by centrifugation over proprietary neutrophil separation media, Polymorphoprep. Heparinised plasma was aspirated from the supernatant. Only samples yielding >95% neutrophil purity were used in experiments (as assessed by Reastain Diff-quik (Reagena, Toivala, Finland) staining of cytospins and analysis using a haemacytometer). Cells were suspended at $10x10^6$/ml in warmed Iscove's modified Dulbecco's medium (IMDM) prior to use.

(h) Phagocytosis assay

i) Core phagocytosis assay

Phagocytosis by neutrophils, and alveolar cells, was assessed by zymosan ingestion. The core assay is described below followed by a description of the variations on the technique used to explore various parts of the mechanism.

Neutrophils derived as described in 2.03 g above were adhered to tissue culture plastic in IMDM containing 1% autologous serum. Cells were exposed for 1 hour

to zymosan particles that had been pre-incubated with 50% autologous serum. Neutrophils were air dried, fixed with methanol and stained with Reastain Diff-Quik. Light microscopy was used to distinguish neutrophils containing ≥2 zymosan particles. Duplicate counts were performed on four randomly selected fields (minimum of 100 neutrophils per field), example fields are shown in figure V-1 (page 144).

ii) Time course experiments
(Chapter 4)

The time course of phagocytosis was explored by exposing cells to zymosan for 5, 10, 15, 30 and 60 minutes, and 'velocity' calculated by change in the percentage of neutrophils undergoing phagocytosis over time.

iii) Testing for defects in opsonisation

(Chapter 4)

Zymosan was opsonised in serum from patients' known to exhibit poor PBN phagocytosis (<40%, see Figure III-1 page 100) or matched volunteers (known to exhibit efficient PBN phagocytosis) for one hour at 37°C. PBNs from healthy volunteers were then exposed to opsonised zymosan at 0.02mg/ml in IMDM with 1% patient or healthy volunteer serum, incubated for 1 hour at 37°C and then washed, stained and counted as per the core protocol above. To demonstrate the need for opsonisation for efficient phagocytosis in this assay, similar experiements were conducted in which zymosan was incubated with heat-inactivated serum (60°C for 60 minutes) or with PBS (i.e. non-opsonised) before being exposed to the PBNs.

iv) Serum inhibitors

(Chapter 4)

Healthy volunteer PBNs were incubated with serum from patients known to exhibit poor PBN phagocytosis (<40%) or from matched volunteers (known to exhibit efficient PBN phagocytosis) at 37°C for one hour. The PBNs were adhered as per the core protocol above, then exposed to zymosan opsonized with either patient serum or healthy volunteer serum for 1 hour at 37°C. Washing, staining and counting were as per the core protocol.

v) Antibody blockade of phagocytic receptors
(Chapter 4)

To test for the role of CD11b, as the major receptor for serum-opsonised zymosan, neutrophils were incubated with murine monoclonal anti-CD11b functional blocking antibody MEM-170. The role of CD16 was examined by blockade using murine monoclonal anti-human CD16 functional blocking antibody 3G8. Control was achieved using isotype murine IgG1.

vi) Inhibitors of phagocytosis
(Chapter 5)
To explore the effects of C5a, a ligand operating via a similar G-coupled receptor (fMLP) and a ligand operating via a receptor coupled to a different type of G-protein (isoproterenol) the ligands were added to the neutrophils during the adhesion phase in the following final concentrations: C5a (100nM), fMLP (100nM), isoproterenol (100μM). A direct activator of phosphoinositol 3 kinase, sub-class IA (PI3K IA) , 740 Y-P was also used in this protocol. In a variation, healthy volunteer PBNs were incubated with either 1μg/ml of S5/1 (Abcam,) - a murine monoclonal antibody known to block CD88 - or with

1μg/ml pre-immune murine IgG1 (Invitrogen) for 30 minutes at 37°C, prior to addition of C5a at concentrations of 1,10 or 100nM (or control) as above. Levels of cAMP following stimulation with C5a, fMLP or isoproterenol in the concentrations noted above were determined in cell lysates using a non-radiographic competitive binding assay (Parameter, RnD Systems).

vii) Intra-cellular signalling down stream of phagocytosis inhibitors

(Chapter 5)

To explore the effects of the above ligands in 2.03 h *vi* on intra-cellular signalling pathways cells were pre-incubated with a number of small inhibitors of key signalling molecules before exposure to the ligand of interest. These molecules included the adenylate cyclase inhibitor SQ22536, the non-isoform specific PI3K inhibitors wortmannin and LY294002, the PI3Kγ inhibitor AS605240, and the PI3Kδ inhibitor IC87114.

vii) GM-CSF and interferon.

(Chapter 5)

To test the ability of certain immune modulators to resurrect C5a-mediated impairment of phagocytosis, cells treated with C5a as per 2.03 h *vi* above were then exposed to GM-CSF, recombinant human interferon γ or control for 30 minutes prior to incubation with opsonised zymosan. Testing of the role of PI3Kδ inhibition in 'treatment' (as opposed to prevention) of C5a-mediated effects was achieved by replacing GM-CSF with IC87114 in this protocol.

ix) Influence of BAL fluid on phagocytosis

(Chapter 4)

To test for any inhibitory effects of BAL fluid from patients with suspected VAP on phagocytosis, neutrophils from healthy donors were incubated with lavage from five patients with suspected VAP with low (<50%) phagocytosis by BAL cells, or from a matched volunteer without discernable respiratory pathology. Cells were incubated for one hour in lavage with 1% autologous (to the neutrophil donor) serum before being exposed to zymosan as per the core protocol. To test for CD88 dependence of the lavage-mediated defect healthy volunteer PBNs were incubated with either 1μg/ml of S5/1 (Abcam,) - a murine monoclonal antibody known to block CD88 - or with 1μg/ml pre-immune murine IgG1 (Invitrogen) for 30 minutes at 37°C, prior to addition of BAL fluid as above.

(i) RhoA activation

(Chapter 5)

For Rho GTPase analysis, neutrophils from healthy donors were adhered to plastic cell culture plates and treated with: control; C5a; IC87114 and C5a; or C5a and GM-CSF, as above, before being exposed to zymosan or vehicle control for 5 minutes (previously established as the time of peak Rho activation). Cells were then washed in ice-cold phosphate buffered saline (PBS) before being lysed with cell lysis solution (Cytoskeleton) containing 10x HALT™ protease and phosphatase inhibitor with 5mM EDTA (Thermo Scientific). Aliquots were taken for protein estimation with the remainder being snap frozen in liquid nitrogen and stored at -80°C before further use. Analysis of samples, equalised for total protein concentration, was by enzyme-linked immunosorbent assay (ELISA; Rho G-lisa™, Cytoskeleton), as per the manufacturer's instructions.

Similar experiments were conducted with neutrophils from critically ill patients (prospective cohort), exposing them to vehicle control or opsonised zymosan.

(j) Actin polymerisation

(Chapter 5)
Healthy donor neutrophils were adhered and treated with: control; C5a; IC81774 and C5a; or C5 and GM-CSF, before being exposed to vehicle control or zymosan for 0 (baseline), 5,10,15 or 30 minutes. Cells were washed in ice-cold PBS before being fixed in 10% formalin for 10 minutes, permeabilised in 0.1% Triton for 3 minutes, washed in PBS and then incubated with FITC-conjugated phalloidin (2u/ml), which irreversibly binds to polymerised, filamentous actin (f-actin) but not to non-polymerised actin (Dancker et al. 1975), for 30 minutes. Cells were washed once more before being suspended in PBS. Analysis of fluorescence was by flow cytometry (FACScan, BD Bioscience). Similar experiments were conducted using neutrophils from critically ill patients (prospective cohort), exposing them to either vehicle control or zymosan for five minutes.

(k) PKB (AKT) phosphorylation

(Chapter 5)
To test for PI3K activity and inhibition, cellular lysates from appropriately treated healthy donor neutrophils were assayed for phospho-PKB. The effect of C5a on phosphorylation of AKT was examined by western blotting. Briefly, $1x10^7$ neutrophils were incubated with PI3K inhibitors (LY294002, AS605240 or IC87114) or vehicle control for 15 minutes prior to stimulation with C5a for 5 or 15 minutes at 37°C. Cells were pelleted at 450g at 4°C for 5 mins, followed by

lysis in ice-cold cell lysis buffer (M-PER™) containing 10X HALT™ protease and phosphatase inhibitor with 5mM EDTA (Thermo Scientific). Following this the lysates were clarified by centrifuged at 14000g for 15 minutes, and the supernatant frozen at -80°C for later analysis.

Samples equalised for protein were mixed in a 1:1 ratio with sample buffer and denatured at 95°C for 5 minutes. Samples were then run on a 12% Pierce Precise protein gel before blotting onto polyvinylidene fluoride (PVDF) membrane. The membrane was blocked for 1 hour in 5% skimmed milk with 0.1% Tween, followed by incubation for 12 hours with a rabbit anti-human phospho-AKT (S473) antibody (R&D systems, Abingdon, UK). The membrane was then washed 3 times in Tris-buffered saline with 0.1% Tween (TBST) before being incubated for 1 hour with horseradish peroxidase-conjugated goat-anti rabbit secondary antibody (R&D systems). The membrane was then washed three times in TBST and exposed to chemiluminescent substrate (Supersignal, Thermo Scientific), washed once in TBST, and exposed to film.

(I) Flow cytometry

i) Isolated neutrophil flow cytometry

(Chapters 3 and 4)
Samples comprised freshly prepared neutrophils from healthy volunteer donors, matched volunteers or VAP cohort patients incubated in IMDM, centrifuged at 300g for 5 minutes at 4°C then resuspended in PBS containing 4% murine serum (with the exception of cells stained for CD88 which were resuspended in 4% caprine serum). Primary antibodies consisted of

- murine monocloncal anti-CD11b, -CD32 (Fcγ receptor II), and -CD64 (Fcγ receptor I) labelled with phycoerythrin (PE), fluorescein-5-isothiocyanate (FITC) and PE respectively;
- murine monoclonal antibody against the activation epitope of CD11b, CBRM1/5, labelled with FITC; and
- murine monoclonal anti-CD88 P12/1, unlabelled.

In the case of anti-CD88, a secondary goat-anti mouse antibody conjugated with alexa-647 was applied.

In parallel, appropriate isotype control antibodies were applied. Cells were washed and fixed with 10% formalin and analysed by flow cytometry (FACSCalibur, BD Bioscience) within 18 hours. Expression was quantified as geometric mean fluorescence.

Stimulation of cells with opsonised zymosan was performed to assess the activation of CD11b. Neutrophils at 500,000/ml were exposed to zymosan opsonised in autologous serum at 0.02mg/ml at 37°C for 30 minutes. Cells were then washed and stained with the activation epitope-specific antibody CBRM1/5 as per the protocol above.

The effects of C5a, patient lavage, C5a followed by GM-CSF and C5a followed by interferon gamma on CD88 expression was assessed by exposing healthy donor neutrophils to these compounds at the concentrations used in the phagocytosis experiments above (section 2.03 h *vii* page 84) for 1 hour at 37°C prior to washing in ice-cold PBS and staining with anti-CD88 antibody as per the above protocol.

ii) Whole blood flow cytometry

(Chapters 5 and 6)

Fifty μl of EDTA-anticoagulated whole blood from healthy donors or critically ill patients (prospective cohort) was incubated with primary, fluorophore-tagged antibodies in the following combinations: anti-human CD11b/FITC; anti-human CD62L (L-selectin)/tricolor (TC); anti-human CD16/TC; anti-human CD64-R-phycoeyrthrin (RPE) and anti-human CD88/Alexa647, or isotype controls, for 30 minutes at 4°C. Cells were fixed with Facslyse and analysed by FACSCalibur (BD Biosciences), gating on neutrophils by forward/side scatter (FSC/SSC) characteristics. Expression was quantified by the geometric mean channel fluorescence (GMF), which is expressed in arbitrary fluorescence units. The effect of C5a exposure on neutrophil CD88 expression was explored by incubating healthy donor neutrophils with varying concentrations of rhC5a (1,10,100 nM and vehicle control) followed by incubation with the fluorophore-labelled anti-CD88 antibody. Monocyte HLA-DR was determined by incubating blood with Quantibrite murine anti-human HLA-DR/R-PE (BD Bioscience), with quantification using periodic calibration of the flow cytometer with Quantibrite beads. The protocol was otherwise as per the neutrophil stains although gates were adjusted to FSC/SSC characteristics of monocytes.

Regulatory T-cells were determined by staining 50μl of whole blood using murine anti-human CD4/FITC and CD25/APC for 30 minutes at 4°C. All reagents were obtained from ebioscience. Red cell lysis buffer was then added to the cells, which were incubated at 21°C for 10 minutes, prior to washing in flow staining buffer (FSB) and resuspending in cell fixation/permeabilisation solution at 4°C for 30 minutes. Cells were then washed twice and resuspended in FSB and incubated with murine anti-human FOXP3/R-PE or R-PE conjugated isotype

control for 30minutes at 4°C prior to washing and resuspending in FSB and analysing on FACSCalibur (BD Bioscience). Analysis was by gating on leucocytes (by FSC/SSC characteristics), identifying the CD4+ve cells and determining the percentage that was positive for CD25+ and FOXP3.

(m) Super-oxide production assay

(Chapter 3)
Neutrophil superoxide anion production by cells obtained from matched volunteers and critically ill patients (VAP cohort) was assayed by a cytochrome C reduction assay, using a stimulus of 100nM platelet activitating factor (PAF) and 100nM N-formyl-methionyl-leucyl-phenylalanine (fMLP).

(n) *Pseudomonas* killing assay

(Chapter 5)
The ability of healthy volunteer neutrophils, pre-incubated in various media, to kill *Pseudomonas aeruginosa* was assayed in the following manner. *P. aeruginosa* strain PA01 was grown for 12 hours in Luria-Bertani (LB) broth at 37°C, before being subcultured into fresh LB Broth at 1:200 dilution and grown for a further 3 hours so as to enter into early logarithmic phase. An aliquot of this early log culture was taken and diluted to an optical density of 0.1 at 595nm in IMDM. This was further diluted by a factor of 1:1000 in IMDM, with the resultant bacterial suspension being used at this concentration. Duplicates of this suspension were diluted $1x10^2$ and $1x10^4$ and plated on LB Agar for overnight incubation to determine baseline colony counts. Simultaneously $1x10^6$ neutrophils suspended in IMDM were incubated with various treatments including BALF supernatant from patients, BALF supernatants from healthy volunteers, C5a at 100nM, or vehicle control, for one hour at 37°C. Following this 1ml of the *P. aeruginosa* suspension was added and the resultant mixture

incubated for a further thirty minutes. Alongside, *P. aeruginosa* was incubated without neutrophils as a positive control. Following this, the neutrophils were lysed with Triton 0.1% and the suspension diluted $1x10^2$ and $1x10^4$ and plated on LB Agar for overnight incubation to determine colony counts.

(o) Transmigration assay

(Chapter 5)
Neutrophils from healthy volunteers were subjected to various treatments detailed below, washed twice and then added to polystyrene inserts (containing pores of 3μm diameter) at 100,000 cells per insert. The inserts were in turn placed in 24-well plates containing a) serum free IMDM and fMLP as a positive control for migration (final concentration 100nM), or b) serum free IMDM alone (as negative control). Plates were incubated at 37°C for one hour. The upper surface of each insert was gently scraped to remove adherent neutrophils then inserts were fixed in methanol and then stained with Diff-Quik. Transmigration was quantified by counting the number of neutrophils on the lower surface of the insert, counting ten randomly selected fields per insert. The pre-treatments were as follows 1) vehicle control, 2) 180nM IC87114 for 15 minutes, 3) 30μM 740 Y-P for 1 hour, 4) 100nM C5a for 1 hour, 5) 180nM IC87114 for 15 minutes followed by 100nM C5a for 1 hour, 6) 100nM C5a for 1 hour followed by 0.3nM GM-CSF for 30 minutes.

(p) Assaying cytokines, chemokines and related molecules

(Chapters 3,4 and 6)
Concentrations of C3a des-arg and C5a des-arg, the breakdown products of complement factors C3a and C5a, were estimated using a cytometric bead array

kit (BD Biosciences). GM-CSF, G-CSF, IL-1β, IL-6, IL-8, IL-10, MCP-1, MIP-1α ,TNF-α and soluble CD62L levels were assessed by the same method. Neutrophil elastase (detected as complexed to native inhibitors) and myeloperoxidase protein concentrations were assayed by ELISA (Hycult Biotech). Due to subsequent non-availability of the CBA assay for C3a-des arg and C5a des-arg, these assays were undertaken by ELISA (BD Bioscience) for the data presented in Chapter 6.

Lavage concentrations of these molecules were corrected for dilution by measuring urea in BALF and plasma (Rennard et al. 1986).

(q) Epithelial cell inflammation assay

(Chapter 3)
A549 cells derived from human type II alveolar epithelial cells (Giard et al. 1973), were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine and 10% fetal calf serum. Confluent cells were washed in DMEM then 500,000 freshly isolated neutrophils were applied in DMEM + L-glutamine with 10% autologous serum. Lipopolysaccharide (LPS) O127:B8, derived from *Escherichia coli,* was added (final concentration 100ng/ml) for 24 hours at 37°C. To distinguish soluble from membrane-mediated effects experiments were repeated with the neutrophils in inserts containing permeable membranes (0.4μm pore size, Transwell), preventing direct contact between neutrophils and A549 cells. IL-6 and IL-8 concentrations in the supernatants from these experiments were assayed using the CBA assay noted in 2.03 p .

(r) Monocyte TNF-α production

(Chapter 3)

Mononuclear cells obtained from the cellular preparation protocol presented in 2.03 g above were plated at 500,000 per well in 24 well tissue culture plates with 10% autologous serum in IMDM. The plates were incubated for 1 hour at 37°C before being washed twice in warmed IMDM to remove non-adherent cells (i.e. lymphocytes). The remaining cells were incubated with IMDM, 10% autologous serum and 10ng/ml LPS O127:B8 derived from *Escherichia coli* for 24 hours at 37°C. Supernatants were assayed for TNF-α by ELISA, and corrected for cellularity by analysis of the total protein content of the wells as assayed by BCA protein assay.

(s) Defining 'immune dysfunction'

(Chapter 6)

Following the finding of a close correlation between phagocytosis and neutrophil surface CD88 expression (Figure IV-12 page 131, and V-27 page 169), CD88 was advanced as a proxy measure for neutrophil dysfunction.

The effect of C5a exposure on neutrophil CD88 expression was explored by incubating healthy donor neutrophils with varying concentrations of rhC5a (1,10,100 nM and vehicle control) followed by incubation with the fluorophore-labelled anti-CD88 antibody. The effect of reduction in CD88 by 10nM C5a, the lowest concentration to significantly affect phagocytosis (Figure V-1 page 144), was examined to determine the cut-off. Neutrophil dysfunction was therefore defined as low neutrophil CD88, specifically a geometric mean CD88 of <250 arbitrary fluorescence units (AFU).

Monocyte 'deactivation' was defined as low monocyte HLA-DR, specifically a geometric mean of <8000 molecules/cell (Meisel et al. 2009).

Regulatory T-cells (T-regs) were identified by CD4, CD25, FOXP3 positivity (Langier, Sade & Kivity 2010) and expressed as a percentage of total CD4 (T_{helper}) cells. 'Elevated' T-regs were defined by reference to the levels found in healthy volunteers as >10% of total CD4 cells (see results section 6.02 k, page 197).

(t) Statistical analysis

Analysis was conducted using Prism (Graphpad Software, La Jolla, CA, USA) and PASW Statistics Version 18 (IBM Corp, Armonk, NY, USA). Contingency tables were analysed by Fisher's exact test (for 2x2) and chi-squared (for >2x2). Difference between two non-normally distributed data sets was compared by Mann-Whitney U test (or Wilcoxon rank sum for paired data). Where more than two data sets were present Kruskal-Wallis ANOVA was used with Dunn's post-hoc test whilst two-way ANOVA (with Bonferroni's post-hoc test) was used for analysis of values over time between two groups. Data summarised by Geometric means were log-transformed before being compared by t-test (for two data sets) or one-way ANOVA with Bonferroni's post-hoc test (for more that two data sets). Binary logistic regression was used to analyse the relationship between immune dysfunctions, and a Cox hazards model was constructed to examine the effects of immune dysfunctions and other clinical variables on acquisition of infection over time. Variables for inclusion in the Cox model were selected through their association with infection acquisition using

univariate analysis (linear regression for continuous/ordinal data and phi co-efficient for categorical data). All variables with a p value of <0.1 on univariate analysis were selected for inclusion. P≤0.05 was considered statistically significant. The assistance of Dr Niall Anderson in developing the cox model is gratefully acknowledged.

(u) Ethical approval

The study was approved by the relevant research ethics committees (RECs).

The work on critically ill patients (VAP cohort) and matched volunteers was approved by the Lothian REC, study numbers LREC/2002/8/19 and 06/S1101/50 respectively.

The work on critically ill patients was approved the Scotland A REC (study number 09/MRE00/19) and healthy volunteers by Lothian REC (study number 08/S1103/38).

Article III. A description of innate immune function and dysfunction in patients with suspected ventilator-associated pneumonia -'septic superposition'.

Section 3.01 Introduction and chapter overview

This chapter is based on a study of patients suspected of having developed the commonest, severe, ICU-acquired infection namely ventilator-associated pneumonia (VAP). As noted in the introduction, VAP is associated with a number of adverse outcomes including prolonged length of stay, increased use of antibiotics and probably increased mortality.

The aims of this study were to characterise the innate immune system's function in the context of suspected and confirmed VAP, examining evidence of both immune dysfunction and activation. The hypothesis being explored was that critically ill patients with suspected VAP would exist in a state of simultaneous immune activation and suppression.

This chapter will explore data from functional assays of immune cells, namely neutrophils, monocytes and alveolar cells (mixed neutrophil/macrophage populations), alongside cell surface and soluble markers of cellular activation and serum cytokine levels.

This chapter contains data from samples collected by Dr Kalliroi Kefala and myself.

Section 3.02 Results

(a) Patients and volunteers

Seventy-three patients with clinically suspected VAP were recruited, one patient did not have recoverable lavage and therefore was excluded from further analysis. Of the remaining 72, 17 (23%) had VAP confirmed by growth of organisms at $>10^4$ CFU/ml. The patients who had clinical features of VAP (i.e. new alveolar shadowing on CXR and evidence of systemic inflammation) but who did not grow organisms at $>10^4$ CFU/ml fell into two groups, those who met the criteria for ARDS/ALI and those who did not (termed 'non-VAP/non-ARDS' group), with 21 patients falling into the ARDS/ALI group and 34 into the non-VAP/ARDS group. Analysis of these three sub-groups allows comparison of patients with infectious pulmonary inflammation (VAP), non-infectious pulmonary inflammation (ARDS/ALI) and a group who were critically ill and ventilated but without a defined pulmonary pathology. 21 age and sex matched volunteers were also recruited to act as a control/reference group. The demographic and clinical details of patients and volunteers are shown in table III-1, the details of the sub-groups are shown in table III-2.

Parameter	Patients	Matched Volunteers	P Value
Age, Mean (range)	58 (26-87)	60 (24-84)	0.89*
% male	64%	79%	0.14[+]
Apache II score, Median (IQR)	22 (20-23)	NA	
Days of ventilation prior to enrolment, Median (IQR)	8 (6-10)	NA	
ICU mortality	35%	NA	
% with surgical diagnosis on admission	52%	NA	
% with ≥ 1 co-morbid conditions	54%	NA	
% receiving immunosuppressive drugs (including corticosteroids)	11%	0%	

Table III-1: Clinical and demographic features of patients with clinically suspected VAP and matched controls.
IQR-inter-quartile range. * p value by t-test, [+] p value by z-test for proportions.

Parameter	VAP subgroup (n=17)	ARDS/ALI subgroup (n=21)	Non-VAP/Non-ARDS subgroup (n=34)	P value
Age, Mean (range)	58 (32-83)	55 (26-80)	59 (25-87)	0.61*
% male	76%	48%	71%	0.73†
Apache II score, Median (IQR)	23 (20-26)	20 (18-22)	22 (20-25)	0.27*
Days of ventilation prior to enrolment, Median (IQR)	8 (6-9)	6 (4-8)	9 (7-14)	0.007‡
ICU mortality	35%	43%	32%	0.73†
% with surgical diagnosis on admission	70%	48%	47%	0.24†
% with ≥ 1 co-morbid conditions	53%	57%	53%	0.95†
% receiving immunosuppressive	12%	19%	6%	0.31†

Table III-2: Demographic and clinical data relating to sub-groups of patients.
IQR-inter-quartile range, P value by *by ANOVA, † by Chi-squared, ‡ by Kruskal-Wallis.

(b) Identification of the defect in phagocytosis.

Peripheral blood neutrophils (PBNs) from patients with suspected VAP demonstrated impaired phagocytosis of serum opsonised particles compared to age- and sex- matched controls. This defect is illustrated in figure III-1 below. Panel A shows pooled results of all patients, whilst panel B shows the results from the 3 sub-groups of patients demonstrating no significant differences between the sub-groups.

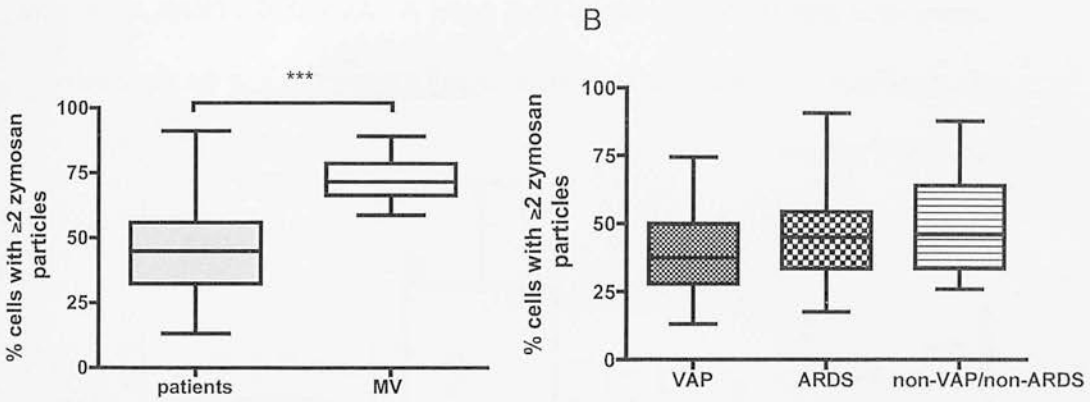


Figure III-1: Phagocytosis by peripheral blood neutrophils (PBNs)

Panel A: **Phagocytosis by PBNs from all patients compared to matched volunteers.** Data are presented as medians, inter-quartile ranges (box) and range (whiskers), *** $p<0.0001$ by Mann-Whitney U-test. Data are derived from 89 subjects (68 patients and 21 MV); in the remaining 4 patients phagocytosis data was not available because of insufficient PBN adherence to tissue culture plates).

Panel B: **Phagocytosis by PBNs of patients by constituent sub-groups.**

Data presented as medians, inter-quartile ranges (box) and range (whiskers), $p=0.162$ by Kruskal-Wallis test. Data are derived from 68 patients (16 VAP, 19 ARDS, 33 non-VAP/non-ARDS).

(c) Phagocytosis by alveolar cells

Alveolar cells recovered from patients with suspected VAP by alveolar lavage were similarly assessed for ability to undertake phagocytosis. A similar defect in phagocytosis was noted, which was all the more striking as the alveolar cells from patients were predominantly neutrophils, whilst those from the matched volunteers were almost exclusively macrophages (see table III-6, page 111). The alveolar macrophage is notably less phagocytic than the PBN, as can be appreciated from the inset in figure III-2, panel A. As with the PBNs, there were no significant differences between alveolar cell phagocytosis in the three sub-groups of patients.

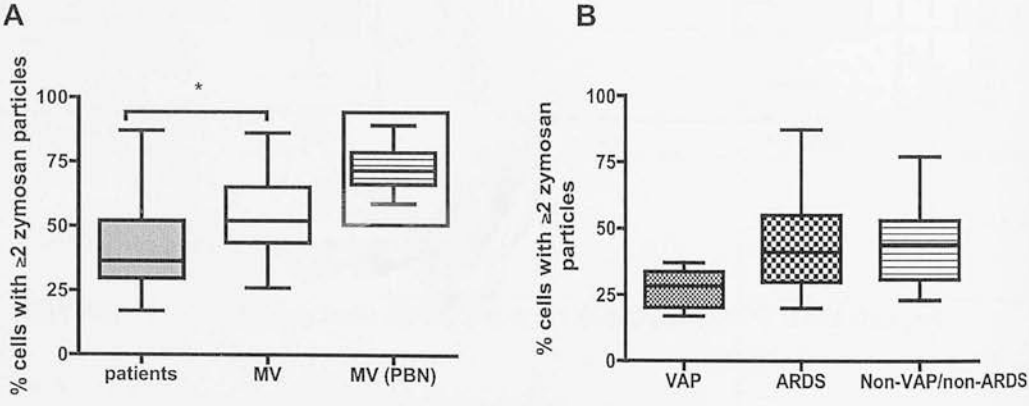


Figure III-2: Phagocytosis by alveolar cells

Panel A: **Phagocytosis by cells retrieved from BALF in patients and in matched volunteers (MV).**

Data are presented as medians, inter-quartile ranges (box) and range (whiskers), * p=0.03 by Mann-Whitney u-test. Data are derived from 44 study participants (33 patients and 11 matched volunteers, i.e. all subjects for whom sufficient numbers of phagocytes were available and adhered to tissue culture plates). Inset shows phagocytosis by PBNs from matched volunteers, equivalent to column 2 in figure III-1, panel A. Presence in this graph is simply to illustrate the relative lack of phagocytic activity of matched volunteer alveolar macrophages.

Panel B: **Phagocytosis by lavage cells of patients by constituent sub-groups.**

Data presented as medians, inter-quartile ranges (box) and range (whiskers), p=0.279 by Kruskal-Wallis test. Data are derived from 33 patients (7 VAP, 12 ARDS, 14 non-VAP/non-ARDS).

(d) Super-oxide production

To examine whether the defects in neutrophil function extended beyond phagocytosis, the ability of neutrophils to produce the reactive oxygen species (ROS) super-oxide ion in response to classic priming and stimulus agents (PAF, and fMLP) was assessed.

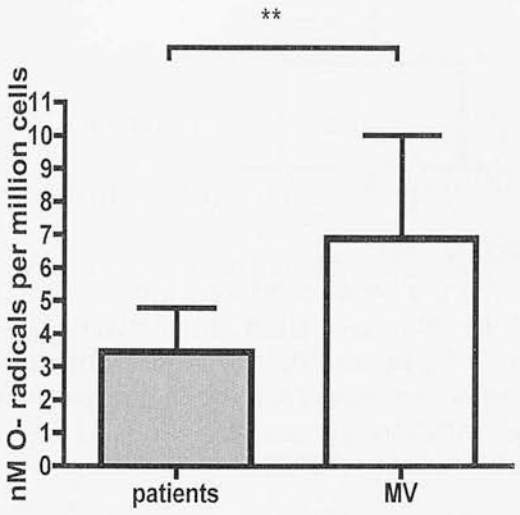


Figure III-3: Production of superoxide radical by neutrophils primed with 100nM PAF and stimulated with 100nM fMLP.
Data shown as median and upper quartile, **p=0.0076 by Mann-Whitney test. Data are derived from 16 patients and 12 matched volunteers, experiments conducted on a consecutive sub-set of study subjects recruited after the initial observations noted above.

The small numbers in each of the sub-groups precluded effective analysis of any differences between sub-groups of patients.

(e) Monocyte response to LPS

Peripheral blood monocytes were assessed for their response to stimulation with *Escherichia coli* (*E. coli*)-derived LPS, in terms of secretion of TNF-α over a 24 hour incubation. This measure of monocyte activity has previously been shown

to predict infectious complications in patients with sepsis (Landelle et al. 2010) and neuro-trauma (Asadullah et al. 1995), and poor response to LPS has been termed 'monocyte deactivation' (Volk et al. 1996).

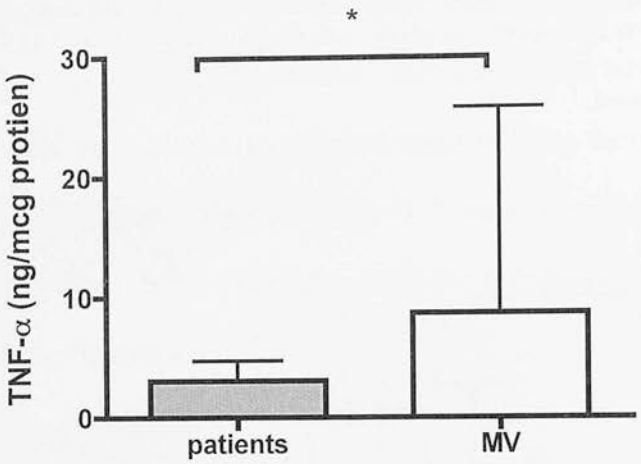


Figure III-4: Supernatant TNF-α levels.
TNF-α corrected for total protein, from peripheral blood monocytes stimulated with 10ng/ml of LPS for 24 hours. Data are shown as medians and inter-quartile ranges, * p=0.04 by Mann-Whitney test. Data derived from 12 patients and 11 matched volunteers. Neutrophil contamination of the mononuclear cell layer following blood preparation reduced the number of patient cells able to be assessed in this manner.

As with the super-oxide assay small numbers prevent meaningful analysis of the patient sub-groups.

(f) Neutrophil activation

Undisturbed neutrophils in the peripheral blood exist in a resting state, however on exposure to a wide variety of stimuli they undergo changes in cell surface confirmation and reactivity to further stimuli in a process termed 'activation' (Condliffe et al. 1996). A number of markers of 'activation' have been demonstrated previously, including up-regulation of the integrin and complement-mediated phagocytosis receptor CD11b, shedding of Fcγ III (CD16)

and extra-cellular release of proteolytic enzymes such as human neutrophil elastase (HNE) and enzymes involved in oxidative killing such as myloperoxidase (MPO) (Berger et al. 1984, Eksioglu-Demiralp et al. 2001, Partrick et al. 1997, Dularay et al. 1990).

Surface expression of CD11b (figure III-5) and CD16 (figure III-6) was determined on neutrophils from patients and matched volunteers, and serum HNE levels were determined by ELISA (Figure III-7). HNE in serum is almost exclusively found in combination with its major inhibitors, including α_1-antitrypsin (Fryksmark et al. 1983), and unsurprisingly no evidence of functional HNE was found in serum (data not shown). Serum MPO levels were also determined by ELISA (Figure III-8) as MPO activity can be variably affected by inhibitors such as ceruloplasmin (Griffin et al. 1999).

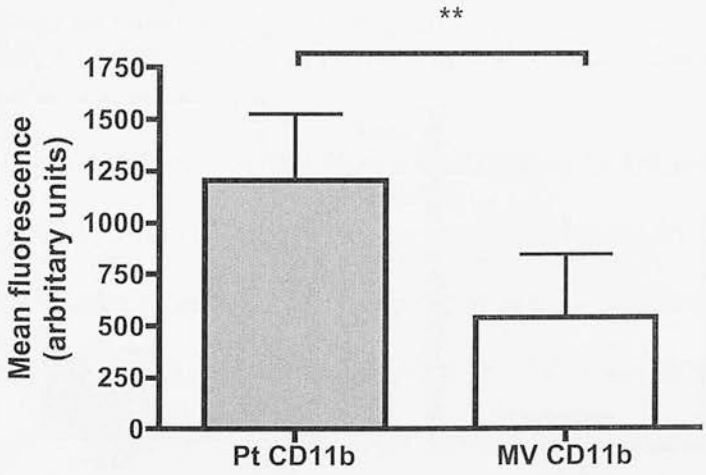


Figure III-5: CD11b expression on PBNs from patients and matched volunteers.
Flow cytometric data shown as geometric means and 95%confidence intervals ** p=0.006 by t-test. n=20 patients and 12 matched volunteers.

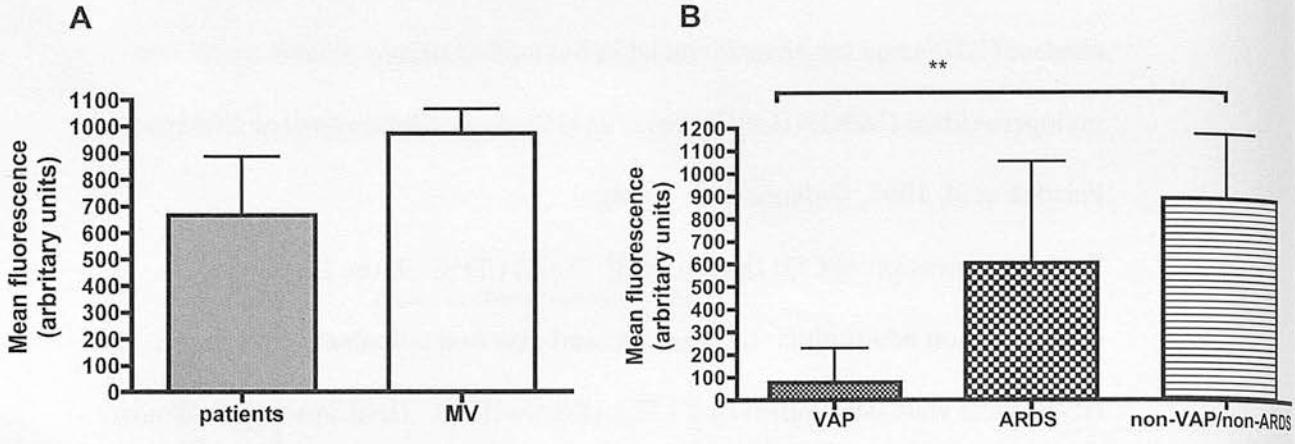


Figure III-6 : Neutrophil surface CD16 expression
Panel A: **CD16 expression (as determined by flow cytometry) on the surface of PBNs from patients and matched volunteers**. Data shown as geometric means and 95% confidence interval p=0.054 by t-test. n=20 patients and 12 matched volunteers
Panel B: **CD16 expression by PBNs from the sub-groups of patients.** . Data shown as geometric means and 95% confidence interval p=0.006 by one-way ANOVA of log-transformed geometric means, **p<0.01 by Bonferroni's post-hoc test. n=4 VAP, 5 ARDS and 11 non-VAP/non-ARDS *** patients.

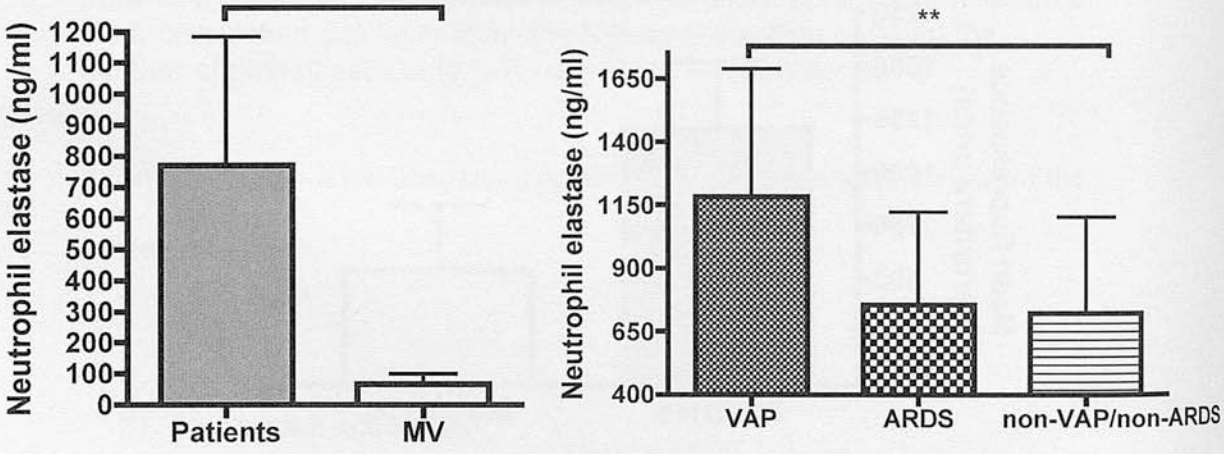


Figure III-7: Serum neutrophil elastase (HNE) levels as determined by ELISA
Panel A: **Serum HNE levels in patients and matched volunteers.** Data shown as medians and upper quartile. ***p<0.0001 by Mann-Whitney test n=72 patients and 21 healthy volunteers.
Panel B: **Serum HNE levels in the sub-groups of patients.** Data shown as medians and upper quartile. p=0.0097 by Kruskal-Wallis, **p<0.001 by Dunn's post-hoc test. n=17 VAP, 21 ARDS and 34 non-VAP/non-ARDS.

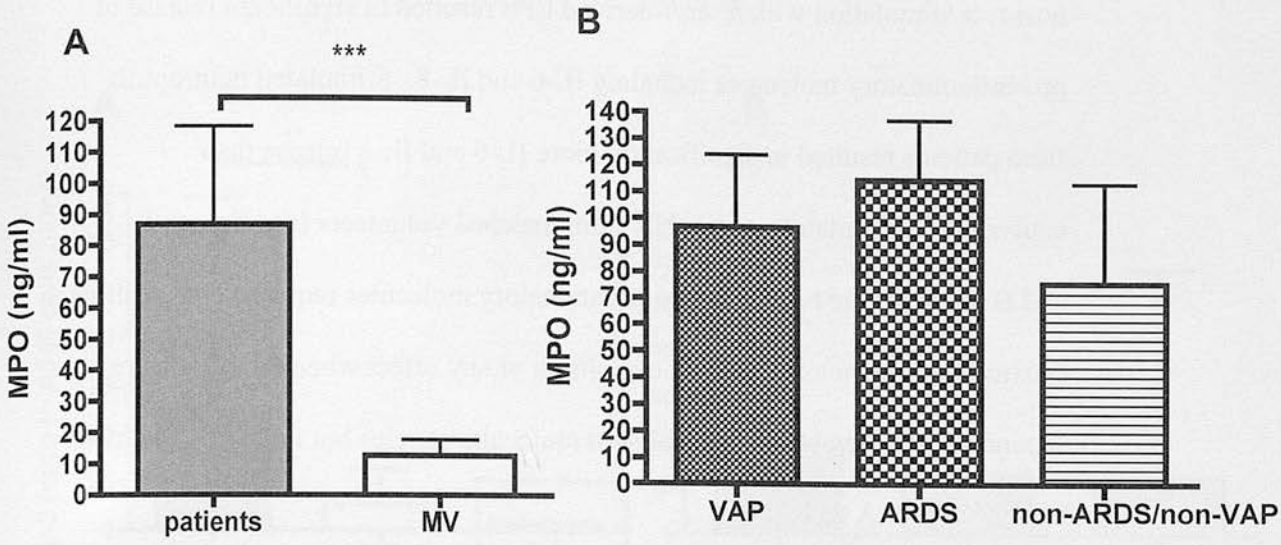


Figure III-8: Serum myloperoxidase (MPO) levels as determined by ELISA

Panel A: **Serum MPO levels in patients and matched volunteers.** Data shown as medians and upper quartile. ***p<0.0001 by Mann-Whitney test n=56 patients and 18 healthy volunteers.

Panel B: **Serum MPO levels in the sub-groups of patients**. Data shown as medians and upper quartile. p=0.2 by Kruskal-Wallis, n=15 VAP, 13 ARDS and 25 non-VAP/non-ARDS

These data demonstrate that despite the defects in the key anti-bacterial functions of phagocytosis and reactive oxygen species production, by standard analyses the patient neutrophils would be considered to be in an 'activated' state. It is also notable that focal lung infection in the form of VAP produced relatively higher levels in some, but not all, peripheral markers of activation.

(g) Neutrophil/Epithelial interactions

Further evidence of the complex, activated phenotype of the peripheral blood neutrophils comes from analysis of their interactions with a cell-line based model of the alveolar epithelium. Neutrophils from patients and matched volunteers were co-cultured with A549 cells, which share some similarities with alveolar type II epithelial cells (Giard et al. 1973). Unstimulated co-cultures did not

produce significantly different results from A549 cells alone (data not shown), however stimulation with *E. coli*-derived LPS resulted in significant release of pro-inflammatory molecules including IL-6 and IL-8. Stimulated neutrophils from patients resulted in significantly more IL-6 and IL-8 release than equivalently stimulated neutrophils from matched volunteers (see figures III-9 A and B below). The release of pro-inflammatory molecules required close cellular proximity, as demonstrated by the abolition of any effect when the cells were separated by a membrane permeable to molecular species but not cells (see insets in figures III-9 A and B).

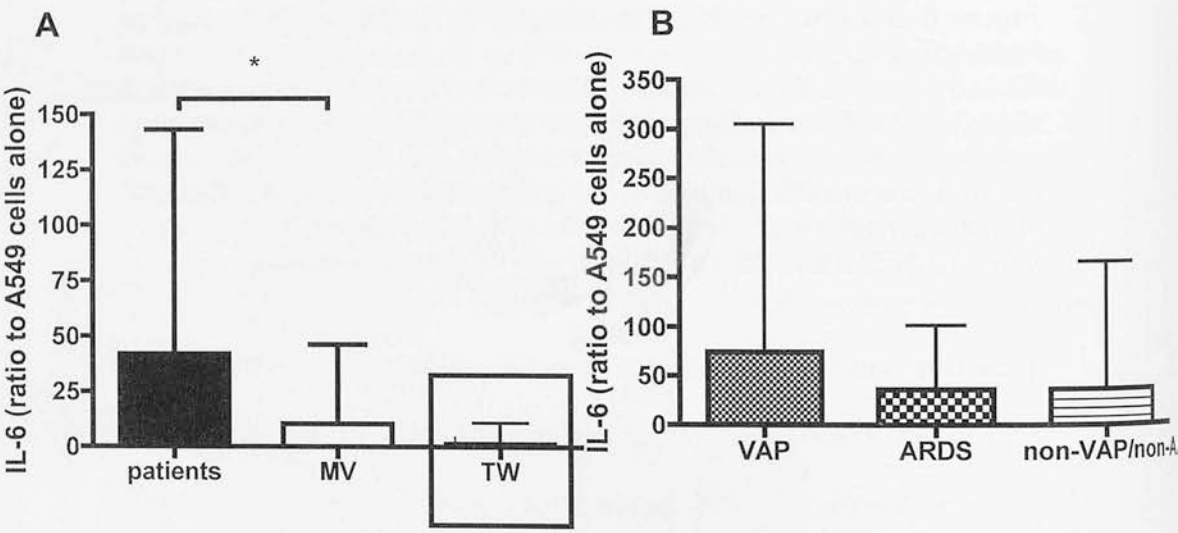


Figure III-9 : IL-6 release by LPS-stimulated neutrophil: A549 co-cultures.
Panel A: **IL-6 release induced by LPS-stimulated neutrophils from patients and matched volunteers (MV).** Data are expressed as a ratio of IL-6 release by stimulated A549 cells to IL-6 release from unstimulated A549 cells. The inset shows IL-6 release when the neutrophils were separated from A549 cells by a transwell membrane with 0.4micron pore size. Data are shown as median and upper quartile, *p=0.017 by Mann-Whitney U-test. Data are derived from 71 study participants (57 patients and 14 MV, i.e. all subjects from whom sufficient neutrophils and A549 cells were available to complete the assay). The transwell data are derived from 12 study participants.
Panel B: **IL-6 release in the patient sub-groups**. p=0.26 by Kruskal-Wallis (n=14 VAP, 15 ARDS and 28 non-VAP/non-ARDS).

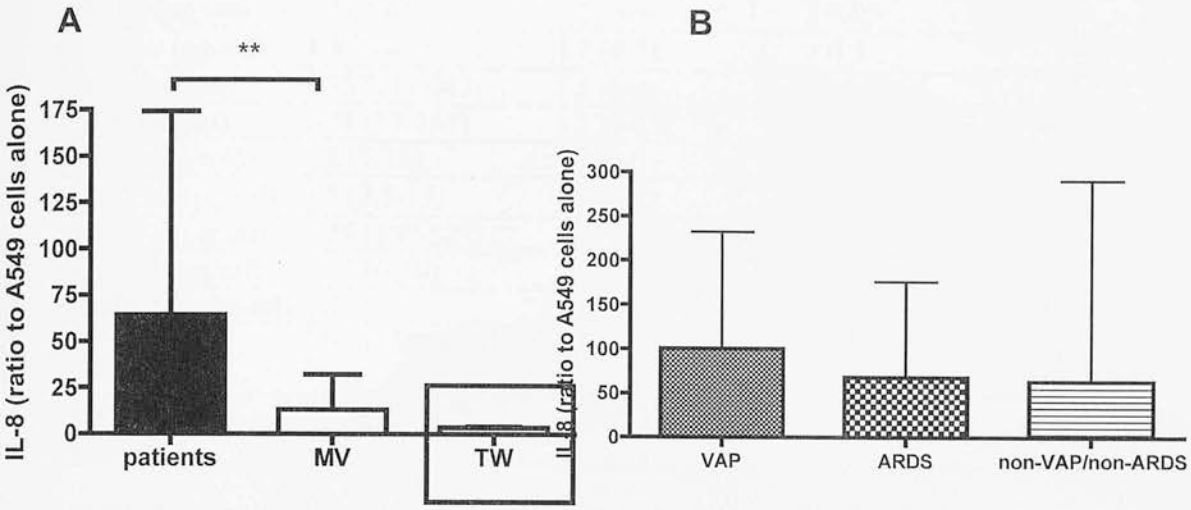


Figure III-10: IL-8 release by LPS stimulated neutrophil: A549 co-cultures
Panel A: **from the same experiments shown in figure III-9 panel A.**
**p=0.0098 by Mann-Whitney.
Panel B: **from the same experiments shown in figure III-9 panel B.**
p=0.86 by Kruskal-Wallis.

(h) Systemic and pulmonary inflammatory molecules

The above data from sections 3.02 f and g demonstrate features associated with neutrophil 'activation'. Alongside this levels of serum inflammatory molecules were also elevated in patients including the pluripotent pro-inflammatory molecule IL-6, anaphylotoxins C3a and C5a, chemotaxins such as IL-8 and MIP-1alpha and granulocyte colony stimulating factor (G-CSF). None of the serum markers distinguished VAP from those without VAP or showed significant variation between the patient sub-groups. To gauge pulmonary inflammation, the same molecules were also measured in BALF. These data are shown in tables

III-3 and III-4 (for patients and matched volunteers) and III-5 and III-6 (patient results divided into the 3 sub-groups).

Serum Cytokine	Patient	Matched volunteer	P value by Mann-Whitney
IL-1β (pg/ml)	4 (3-6)	2 (0-5)	0.05
TNF-α (pg/ml)	3 (0-4)	2 (0-5)	0.5
IL-6 (pg/ml)	107 (41-287)	3 (0-4)	<0.0001
IL-8 (pg/ml)	78 (37-265)	3 (0-12)	<0.0001
IL-10 (pg/ml)	9 (5-18)	0 (0-3)	<0.0001
MIP-1α (pg/ml)	5 (2.5-12)	2 (0-3)	0.0003
MCP-1 (pg/ml)	294 (97-527)	62 (44-552)	0.2
G-CSF (pg/ml)	17 (6-40)	4 (0-19)	0.004
GM-CSF (pg/ml)	3 (2-4)	2 (0-5)	0.34
C3a des-arg (ng/ml)	606 (388-847)	346 (216-400)	0.0006
C5a des-arg (ng/ml)	90 (59-141)	36 (25-43)	<0.0001

Table III-3: Serum cytokine and anaphylatoxin levels amongst patients and matched volunteers.
Data shown as median and inter-quartile ranges.

Alveolar Cytokine	Patient	Matched volunteer	P value by Mann-Whitney
IL-1β (pg/ml)	11 (0-120)	0 (0-1.6)	<0.0001
TNF-α (pg/ml)	0 (0-5)	0 (0-0)	0.12
IL-6 (pg/ml)	134 (13-464)	0 (0-6)	<0.0001
IL-8 (pg/ml)	602 (83-4227)	57 (27-581)	0.004
IL-10 (pg/ml)	1 (0-14)	0 (0-0)	0.02
MIP-1α (pg/ml)	6 (1-15)	2 (0-13)	0.07
MCP-1 (pg/ml)	222 (49-985)	21 (0-54)	<0.0001
G-CSF (pg/ml)	37 (7-138)	6 (0-25)	0.009
GM-CSF (pg/ml)	2(1-9)	2 (0-4)	0.23
C3a des-arg (ng/ml)	15 (4-130)	1 (0-1)	<0.0001
C5a des-arg (ng/ml)	6 (0.5-30)	1 (0-2)	0.005
Human neutrophil elastase (ng/ml)	366 (136-3096)	21 (3-360)	0.006
neutrophils	2.1 (0.3-6.4)	0 (0-0.03)	<0.0001
macrophages	1 (0.2-2.8)	2.9 (1.4-3.9)	0.03

Table III-4: Alveolar cytokine, anaphylatoxin and inflammatory cell levels amongst patients and matched volunteers
Data shown as median and inter-quartile ranges, (corrected for dilution by urea method (Rennard et al. 1986).

Serum Cytokine	VAP	ARDS/ALI	Non-VAP/Non-ARDS	P value by Kruskal-Wallis
IL-1β (pg/ml)	4 (1-7)	4 (0-5)	4 (0-5)	0.9
TNF-α (pg/ml)	3 (1-5)	4 (0-5)	3 (0-4)	0.9
IL-6 (pg/ml)	106 (55-174)	127 (46-262)	100 (27-439)	1.0
IL-8 (pg/ml)	80 (62-324)	90 (47-277)	50 (27-255)	0.2
IL-10 (pg/ml)	8 (5-15)	10 (5-35)	8 (5-14)	0.6
MIP-1α (pg/ml)	5 (0-19)	9 (3-14)	8 (4-50)	0.6
MCP-1 (pg/ml)	343 (136-868)	326 (187-535)	226 (51-468)	0.3
G-CSF (pg/ml)	29 (7-69)	11 (7-25)	16 (6-31)	0.2
GM-CSF (pg/ml)	2 (0-4)	3(2-3)	2 (1-4)	0.4
C3a des-arg (ng/ml)	584 (496-913)	710 (370-972)	612 (326-826)	0.8
C5a des-arg (ng/ml)	113 (62-169)	121 (60-186)	74 (43-130)	0.3

Table III-5: Serum cytokine and anaphylatoxin levels amongst patient sub-groups.
Data shown as median and inter-quartile ranges.

Alveolar Cytokine	VAP	ARDS/ALI	Non-VAP/Non-ARDS	P value by Kruskal-Wallis
IL-1β (pg/ml)	99 (21-697)	1 (0-10)	4 (0-166)	0.0003
TNF-α (pg/ml)	1 (0-18)	0 (0-3)	0 (0-7)	0.4
IL-6 (pg/ml)	266 (105-503)	118 (29-431)	53 (1.7-498)	0.2
IL-8 (pg/ml)	6172 (2060-10742)	430 (188-847)	168 (64-931)	<0.0001
IL-10 (pg/ml)	0 (0-9)	1 (0-4)	1 (0-33)	0.7
MIP-1α (pg/ml)	51 (14-269)	3 (0-20)	4 (1-27)	0.004
MCP-1 (pg/ml)	293 (94-554)	558 (170-1787)	78 (12-573)	0.02
G-CSF (pg/ml)	107 (38-383)	10 (5-32)	30 (6-164)	0.009
GM-CSF (pg/ml)	2(0-14)	1 (0-6)	3 (1-12)	0.4
C3a des-arg (ng/ml)	11 (3-227)	8(4-348)	20 (4-105)	1.0
C5a des-arg (ng/ml)	3 (1-32)	3(2-21)	8 (0-35)	0.8
Human neutrophil elastase (ng/ml)	2708 (675-13293)	311 (71-455)	315 (143-819)	0.003
Neutrophils	4.6 (0.2-17)	2.7 (1.1-11)	0.8 (0.03-4.8)	0.09
Macrophages	1.1 (0.4-3.2)	0.5 (0.15-1.5)	1.4 (0.2-3.9)	0.5

Table III-6:Alveolar cytokine, anaphylatoxin and inflammatory cell levels amongst patient sub-groups.
Data shown as median and inter-quartile ranges, (corrected for dilution by urea method (Rennard et al. 1986).

Section 3.03 Chapter summary and discussion

Critically ill patients have a wide range of precipitating illnesses and insults, but often end up with what appears to be a common phenotype. This consists of failure of several organ systems, often away from the site of the initial insult (e.g. respiratory and renal failure in pancreatitis or following major haemorrhage) and are attributable to secondary damage (Singh, Evans 2006). As noted in the introduction, this secondary damage is mediated in part by the patient's own immune system, following inappropriate systemic activation (Rittirsch, Flierl & Ward 2008). At an organism level this manifests as signs of systemic inflammation, with elevated temperature and signs of increased metabolic demand (increased cardiac output, respiratory rate), accompanied by increases in peripheral blood neutrophil counts, acute phase proteins and other molecular species associated with a pro-inflammatory response. This syndrome has been termed systemic inflammatory response syndrome (SIRS) (American College of Chest Physicians/Society of Critical Care Medicine 1992).

Evidence of this systemic inflammatory response is demonstrated in the data shown above, with 'activated' neutrophils releasing high levels of the major proteolytic enzyme HNE, a species that has been associated with the tissue damage seen in critical illness (Sakamoto et al. 2010). Similarly the neutrophils exert a pro-inflammatory effect on epithelial cells in LPS-stimulated co-cultures, and the serum demonstrates elevated levels of pro-inflammatory species. Although all patients demonstrate evidence of this systemic inflammatory response, there are differences between the sub-groups. The patients with VAP

exhibit higher levels of serum elastase and diminished levels of neutrophil surface CD16 suggesting a higher degree of activation in these patients. However the lack of differences between patient sub-groups in other measures of inflammatory activity suggest that 'activation' is not a simple 'all or nothing' process and that different aspects are differentially regulated.

Strikingly, alongside this hyper-inflammation, there is a marked degree of hypo-activity. Key neutrophil functions of phagocytosis and stimulated production of ROS are significantly depressed compared to age and sex matched controls. Both these processes are of crucial importance for the removal of bacteria and fungi (Julia et al. 1991, Shiohara et al. 2009), and individuals with in-born defects in these functions are at increased risk of infection (Kuijpers et al. 1997, Winkelstein et al. 2000).

The finding of neutrophil hypo-function in critical illness is not a new one (Palmer, Bornside & Nance 1982), however it has been suggested that this might result from sampling the peripheral pool, when one may expect the most active neutrophils to be found in inflamed tissue and hence not recoverable peripherally. This suggestion is refuted by the finding that phagocytosis is equally impaired in cells recovered from the inflamed pulmonary compartment.

Alongside these dysfunctional neutrophils is found evidence of hypo-activity in another key immune cell, the monocyte. Monocytes from patients failed to mount an effective response to stimulation with LPS, so called 'monocyte deactivation' (Volk et al. 1996). TNF-α is of crucial importance to the removal of potentially infectious organisms (Moore et al. 2005), and perhaps

unsurprisingly anti-TNF therapies are associated with increased risk of infection (Bongartz et al. 2006).

It is of note that whilst the serum profile of cytokines did not display significant differences between patients with and without confirmed VAP, the pulmonary compartment showed a marked difference. The presence of infective organisms at sufficient concentration (i.e. above the 10^4 colony forming unit cut off established for lavage based diagnosis of VAP) is associated with an intense pulmonary inflammation as indicated by elevated levels of IL-1β, IL-8, MCP-1, MIP1-A, G-CSF, and HNE with a trend towards greater neutrophilia. Several of these molecules show potential as diagnostic biomarkers (Conway Morris et al. 2010). However despite this intense inflammation, alveolar neutrophil function remains poor (figure III-2B), and indeed it is tempting to speculate that it is this very failure of neutrophil function which permits pathogen growth and the resultant inflammatory response.

It is interesting to note that whilst the patients with VAP displayed an intense pulmonary inflammation, those with ARDS did not. This contrasts with previous work which shows an early rise cytokines such as IL-8 in ARDS (Donnelly et al. 1993). The reason for this disparity is likely to be the timing of sampling, with the current cohort being recruited at a median of 8 days into their ICU stay, and so those with ARDS are likely to be at a later stage of the disease than that sampled in previous studies.

The picture of the innate immune system in this group of patients, i.e. critically ill patients with clinically suspected VAP, is a complex phenotype displaying features of both hyper-activity and hypo-activity simultaneously. To borrow a term from particle physics, the innate immune system appears to be in a condition of 'super-position', simultaneously occupying two distinct and contrasting states. As mentioned above the classical description of critical illness is one of hyper-inflammatory SIRS. A hypo-inflammatory component of the phenomenon has been recognised for some time and indeed the term compensatory anti-inflammatory response syndrome (CARS) has been coined to describe this (Bone 1996). However the standard description of the temporal relationship between SIRS and CARS is a bi-phasic process, with hyper-inflammation predominating at the start of critical illness and immuno-compromise occurring as the disease progresses (Hotchkiss, Karl 2003). More recently it has been suggested that this description is rather simplistic and that there is an alternating pattern of immuno-compromise and hyper-inflammation (Wang, Deng 2008), however the data presented above shows that both states can occur simultaneously. This produces the worst possible combination, with immune-mediated host damage occurring at the same time as increased vulnerability infecting organisms, resulting in either persistence of a primary infection or allowing secondary infection to supervene. From the evidence presented above, it is clear that secondary infection in the form of ventilator-associated pneumonia can further enhance inflammation and tissue damage, so adding to a vicious circle of host damage and proliferation of infecting organisms. This data supports the initial hypothesis that critically ill patients

with suspected VAP would exist in a state of simultaneous immune activation and suppression.

The results of this chapter raise the question as to what is mediating the various immune effects, and to what extent the pro-inflammatory mediators drive the dysfunction seen. Clearly these are potentially massive questions, and therefore attention will focus on one specific immune dysfunction namely phagocytosis by neutrophils which forms the basis for the next two chapters.

Article IV. An exploration of the mediator(s) influencing the defect in phagocytosis noted in patients with suspected ventilator-associated pneumonia

Section 4.01 Introduction

In the examination of the innate immune system in suspected VAP in chapter 3, there was clear evidence of both immune dysfunction and activation. One of the most striking findings was that of diminished phagocytosis by neutrophils, as this defect was accompanied by increased neutrophil enzyme release, elevated cell-surface markers of activation and enhanced pro-inflammatory interactions with epithelial cells. The defect in phagocytosis, as noted in the Introduction (section 1.07, page 59) a key antibacterial function, therefore seems an intriguing target for further investigation.

It has been noted in the Introduction (section 1.07 b, page 61) that phagocytosis is not a single process, but rather a group of processes which all result in the internalisation of particles. The ingestion of serum-opsonised zymosan particles is a complement-mediated response (reviewed in Blom, Hallstrom & Riesbeck 2009) which involves binding of C3b-opsonised particles to MAC-1/CD11b/18 (reviewed in Lee, Harrison & Grinstein 2003), which triggers intracellular signalling pathways which result in the activation of RhoA (Caron, Hall 1998), polymerisation of actin (Kaplan 1977) and internalisation of the particle into a phagosome.

Defects in phagocytosis could therefore be mediated at several levels within this pathway. If the patient serum was deficient in complement then deficient opsonisation may occur. It could be that cell surface CD11b expression is diminished, or that it fails to activate effectively, or there may be an as yet uncharacterised serum component which blocks the binding of particles to the receptor. A further possibility could be that a mediator, acting via non-phagocytic receptors, is able to impair the intra-cellular machinery required for effective phagocytosis. Finally, it is possible that the neutrophils from patients are dead or dying and therefore incapable of any effective functions.

The aim of this chapter, in conjunction with the following chapter 5, is to explore the second principle hypothesis set out in the introduction, namely that human neutrophil dysfunction in critical illness is driven by excessive pro-inflammatory stimulation by C5a, i.e. the penultimate potential mechanism set out in the paragraph above. However as the other potential mechanisms are equally plausible, they have will also be explored in this chapter.

This chapter starts with a characterisation of the phagocytosis assay used, undertaken using neutrophils from healthy donors. There follows an exploration of each of the potential mechanisms in neutrophils from patients with suspected VAP, or using patient serum applied to healthy donor neutrophils. The final potential mechanism (namely that patient neutrophils are dead or dying) is not directly addressed in this chapter, but will be explored in chapter 5. As noted in chapter 3, alveolar cells also display impaired phagocytosis in patients with

suspected VAP. This chapter ends with an examination of this defect and whether or not this displays any signature of C5a mediation.

Section 4.02 Results

(a) Validation and characterisation of phagocytosis assay

To validate and characterise our model of complement-mediated neutrophil phagocytosis four experiements were undertaken. First the role of non-specific/scavenger receptor versus opsonin-assisted phagocytosis was explored. Neutrophils from healthy donors were incubated with zymosan that had been either opsonised in serum or incubated in PBS (Figure IV-1 below).

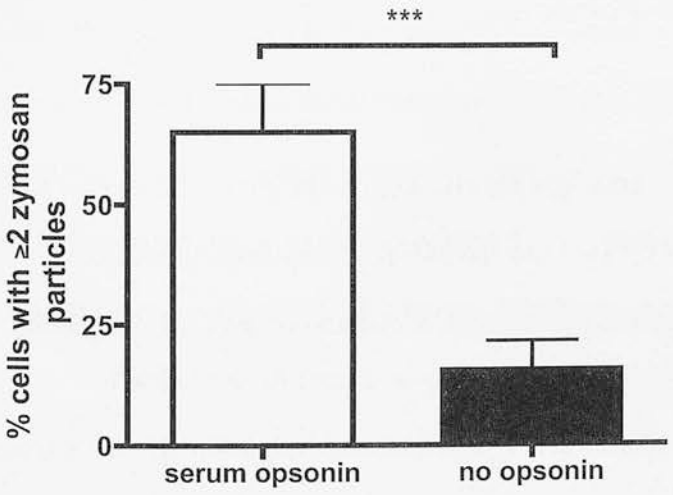


Figure IV-1: The effect of serum opsonisation on phagocytosis of zymosan particles.
Data shown as median and upper quartile, n=6 individual donors. *** p=0.0002 by Mann-Whitney test.

The second validation experiment examined the effect of heat-treating the serum (60°C for 60 minutes) to inactivate complement (Gordon 1953). The results of this are shown in figure IV-2 below.

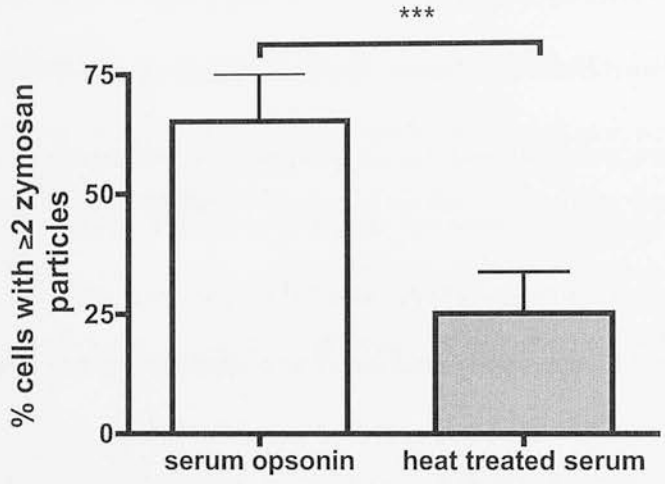


Figure IV-2: The effect of heat-treating serum on phagocytosis of zymosan particles.
Data shown as median and upper quartile, n=6 individual donors. *** $p<0.0001$ by Mann-Whitney test.

The third experiment examined the effect of blocking one of the major C3b receptors, CD11b, which is a key receptor for complement-mediated phagocytosis (Mantovani, Rabinovitch & Nussenzweig 1972). The results are shown in figure IV-3.

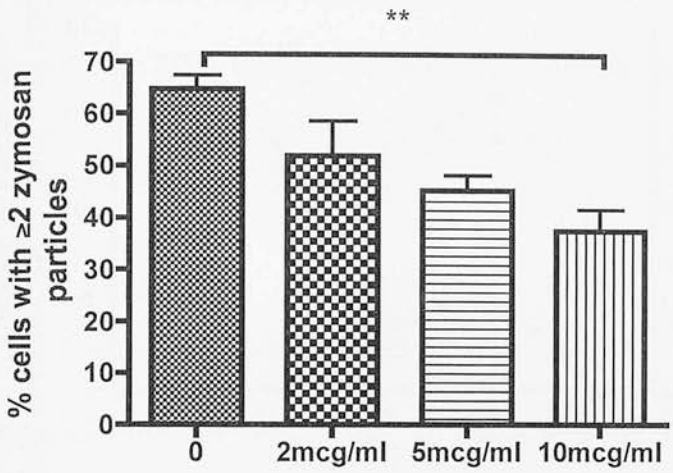


Figure IV-3: The effect of pre-incubation of neutrophils with CD11b blocking antibody (M1/70) on phagocytosis.
n=4 individual donors, data shown as median and upper quartile. P=0.004 by Kruskal-Wallis, ** P<0.01 by Dunn's post hoc test.

The fourth experiment was to determine the time course of phagocytosis. Neutrophils from the same donor were exposed to zymosan for 5, 10, 15, 30 and 60 minutes. The maximum velocity of phagocytosis (% change in proportion of cells with zymosan particles in) was achieved at 5 minutes, and maximum phagocytosis was achieved by 30 minutes. Simultaneously, the level of polymerised actin was determined at each time point using neutrophils from the same donors. The results of these two assays are shown in figure IV-4A below. Figure IV-4B shows example data of actin polymerisation as determined by flow cytometry.

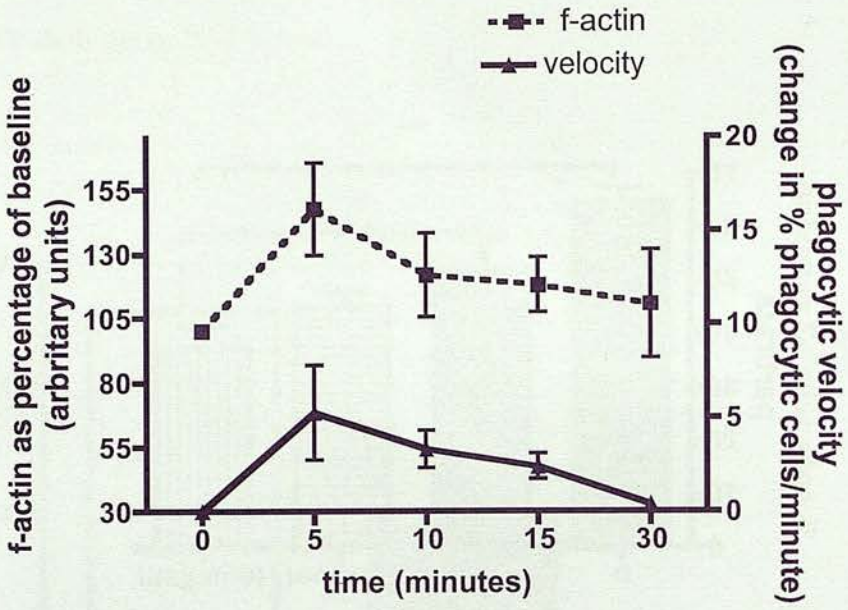


Figure IV-4: Velocity of phagocytosis of zymosan particles, and change in polymerised actin (f-actin) content of neutrophils exposed to zymosan. Data shown as mean and SEM from n=3 individual donors.

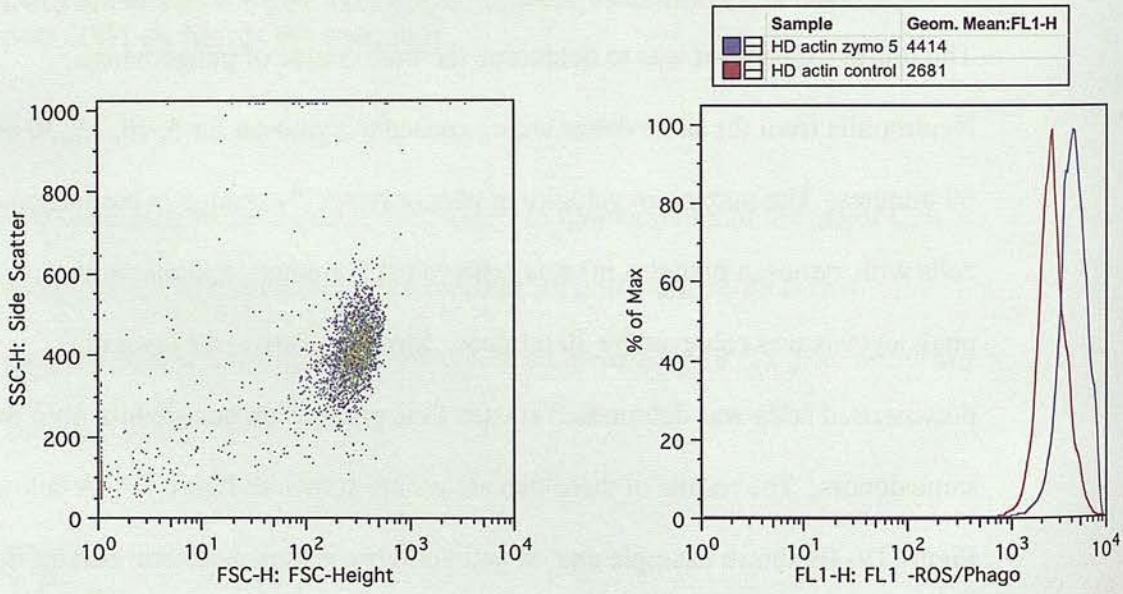


Figure IV-5: Example data of the actin polymersation experiment. The right-hand panel shows the forward/side scatter characteristics of the neutrophils, and the left-hand shows the FL-1 channel fluorescence (indicating degree of phalloidin staining) in unstimulated cells (red) and those exposed to zymosan for five minutes (blue). The geometric mean fluorescence for each is displayed in the box above.

The results above support the assay used being heat labile opsonin-dependent, predominantly mediated by the C3b receptor CD11b/18 and showing actin polymerisation and depolymerisation over a similar time course to the rate of phagocytosis. These features are consistent with zymosan phagocytosis being predominantly complement mediated.

(b) Examining the defect in patient phagocytosis

Having identified the defect in phagocytosis in patients' neutrophils noted in chapter 3, the next step was to try and identify the causative factor(s) and mechanism by which they worked. As set out in the introduction above, the process of efficient phagocytosis requires several stages. These are namely opsonisation, ligation, internalisation and phago-lysosome formation. The following possible mechanisms were explored: 1) defective opsonisation by patient serum; 2) defective expression of phagocytic receptors; 3) defective activation of phagocytic receptors; 4) serum-based inhibitor of phagocytosis.

(c) Opsonising capacity of serum

To examine whether the defect in phagocytosis was due to a failure of opsonisation, healthy volunteer neutrophils were exposed to zymosan particles opsonised in serum from patients and heterologous healthy volunteers (to control for any effect seen being due to serum being heterologous). The neutrophils were adhered in 10% autologous serum and then exposed to zymosan in serum

free IMDM (to prevent opsonisation occurring with autologous serum). No defect in phagocytosis could be detected (figure IV-6).

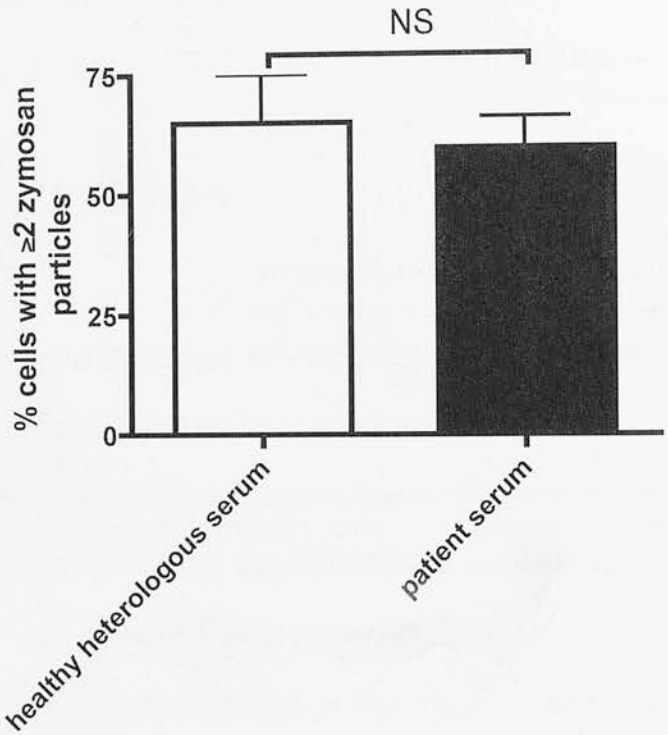


Figure IV-6: The effect of patient serum opsonisation on phagocytosis
The experiment shown invovlved incubating healthy volunteer neutrophils with zymosan opsonised in heterologous serum from healthy volunteers and patients with poor (<40%) phagocytosis. Data shown as median and upper quartile. n=6 healthy donors. P=0.61 by Mann-Whitney test.

(d) Expression of phagocytic receptors by patient neutrophils

Expression of four of the major phagocytic receptors were determined by flow cytomtery, namely the C3b receptor CD11b and the Fcγ receptors I, II and III

(CD64, 32 and 16 respectively). Table IV-1 below illustrates the differences in geometric mean fluorescence relative to matched volunteers.

Receptor	Patient Geometric mean (95% CI)	Matched Volunteer geometric mean (95% CI)	P Value (t-test of log-transformed geo-means)
CD11b	982 (696-1385)	516 (327-813)	0.02
CD16	388 (202-745)	914 (724-1154)	0.04
CD32	33 (22-52)	48 (37-61)	0.3
CD64	33 (22-51)	8 (6-11)	<0.0001

Table IV-1: Expression of phagocytic receptors on PBNs from patients and matched volunteers.
Data from 21 patients and 14 matched volunteers. Data shown as geometric mean and 95% confidence interval, p value by t-test of log-transformed geometric means.

(e) Role of CD16 in neutrophil phagocytosis.

Following the finding in table IV-1 above of diminished CD16 expression on patient neutrophils, its role in the phagocytosis assay was assessed by blockade with an murine monoclonal anti-CD16 antibody 3G8(Rosenberg, Melnicoff & Wilding 1985). As shown in figure IV-7 below, there was no reduction in phagocytosis following CD16 blockade, in marked contrast to the effect seen with CD11b blockade shown in figure IV-3 above.

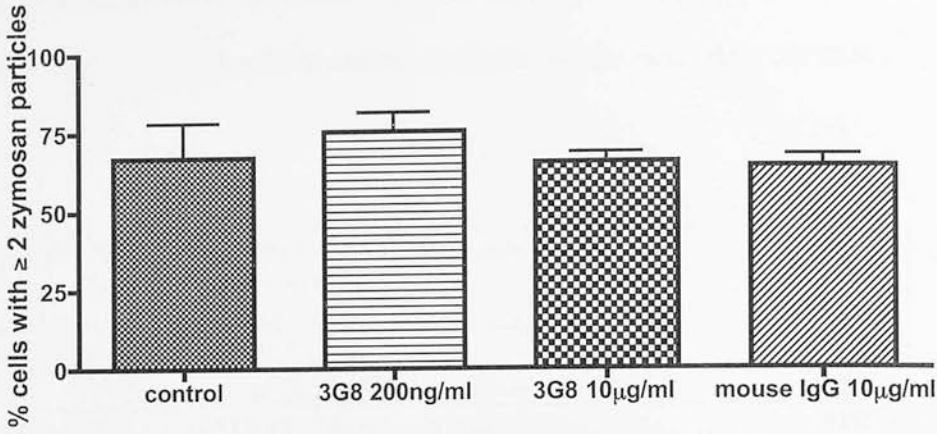


Figure IV-7: The effect of pre-incubation with murine anti-CD16 antibody 3G8 or murine IgG1 isotype control on phagocytosis. Results shown as median and upper quartile range from n=3 individual donors, p=0.8 by Kruskal-Wallis test.

(f) CD11b activation following exposure to zymosan.

Exposure of neutrophils to zymosan particles induces both up-regulation of CD11b and increased exposure of the activation epitope (Diamond, TD 1993). Table IV-2 shows the effect of both opsonised and non-opsonised zymosan exposure on CD11b expression and CD11b activation in patients and matched volunteers. As can be seen although there is a trend towards increased basal CD11b expression by patient neutrophils, this is not reflected in an increase in the amount of *activated* CD11b present before exposure to zymosan. The loss of significance in the difference between patients and matched volunteer total CD11b (cf table IV-1 above) is probably due to the reduction in numbers of individuals examined. Following exposure to both opsonised and non-opsonised zymosan both patients' and volunteers' neutrophils up-regulated the amount of activated CD11b relative to basal conditions, although there were no significant differences between the groups of participants for each stimulating condition.

Volunteer neutrophils were able to up-regulate total CD11b on stimulation with both forms of zymosan, in contrast the patient neutrophils were not able to significantly elevate CD11b expression above basal conditions. However the apparent failure of CD11b up regulation by patient neutrophils is a relative one, due to the already higher basal levels, suggesting that the patient cells had already achieved near maximal CD11b expression.

		Patients	**Matched volunteers**	**P value**
Unstimulated	CD11b	1157 (845-1584)	662 (359-1219)	0.06
	Activated CD11b	10 (8-12)	12 (8-19)	0.2
Stimulated with non-opsonised zymosan	CD11b	1873 (1211-2896)	1578 (588-4234)	0.3
	Activated CD11b	26 (16-35)	32 (27-38)	0.3
Stimulated with opsonised zymosan	CD11b	1975 (1312-2973)	1468 (899-2398)	0.5
	Activated CD11b	31 (19-43)	26 (21-31)	0.95

Table IV-2: CD11b expression and activation in neutrophils from patients and healthy volunteer neutrophils in basal and stimulated conditions
Stimuli were non-opsonised and serum opsonised zymosan. N=18 patients and 7 healthy volunteers, p value in right hand column by t-test of log-transformed geometric mean fluorescence, assessed using flow cytometry.

	Basal	Non-opsonised	Opsonised	P value
Patients – CD11b	1157 (845-1584)	1873 (1211-2896)	1975 (1312-2973)	0.78
Patients – activated CD11b	10 (8-12)	26 (16-35)***	31 (19-43)***	<0.0001
Matched Volunteers-CD11b	662 (359-1219)	1578 (588-4234)*	1468 (899-2398)*	0.02
Mactched Volunteers-activated CD11b	12 (8-19)	32 (27-38)**	26 (21-31)**	<0.0001

Table IV-3: CD11b expression and activation in neutrophils from patients and healthy volunteer neutrophils in basal and stimulated conditions

Stimuli being non-opsonised and serum opsonised zymosan). N=18 patients and 7 healthy volunteers, p value in right hand column by one way ANOVA of log-transformed geometric mean fluorescence, assessed using flow cytometry. *p<0.05, *** P<0.001 by Bonferroni's post-hoc test compared to unstimulated conditions. Note, this is the same data as presented in table IV-2, arranged to show comparisons within participant groups. The tables have been rearranged for clarity of statistical analysis.

(g) Serum-based factor inhibiting phagocytosis independent of opsonisation.

To examine a possible serum-based inhibitor of phagocytosis, healthy donor neutrophils were incubated with serum from patients with poor phagocytosis

(<40% of cells phagocytic). The results shown in Figure IV-8 below indicate no inhibitory effect of serum on phagocytosis.

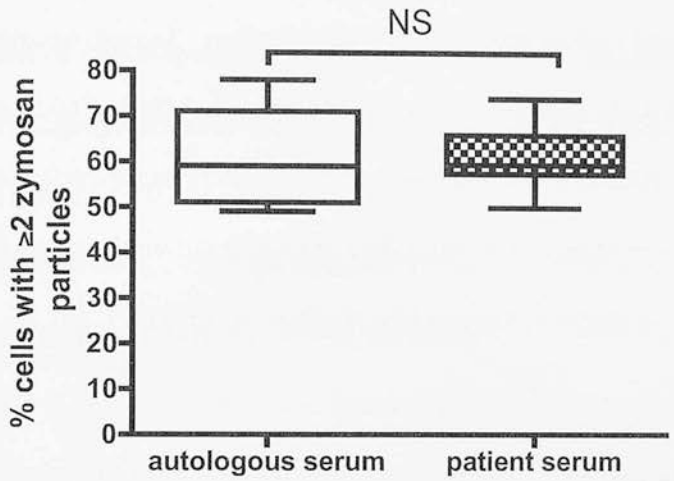


Figure IV-8: Effect of patient serum on neutrophil phagocytosis. Phagocytosis by healthy volunteer neutrophils incubated in autologous serum or pooled serum from 4 patients with poorly phagocytic (<40%) neutrophils. Data shown as median, interquartile (box) and range (whiskers) from n=8 healthy donors, p=0.64 by Wilcoxon rank sum test.

(h) Complement split product C5a as a mediator of impaired phagocytosis – measuring C5a exposure.

The anaphylatoxin C5a is largely generated by processes down-stream of C3 activation, which releases C3a and b (reviewed by Ward, 2004). C5a, and indeed C3a, are rapidly converted to the considerably less potent 'des-arg' forms by carboxypeptidase-mediated removal of the arginine residue from the carboxyl end of the proteins (Campbell et al. 2002). Furthermore C5a is removed from the circulation by binding to CD88 and being internalised by a variety of cells including neutrophils (Chenoweth, Hugli 1978), giving it a plasma half-life of 2 to 3 minutes (Webster, Larsen & Henson 1982). Therefore a simple measure of

plasma C5a concentration is unlikely to give an accurate impression of *in-vivo* exposure of neutrophils to C5a.

Two proxy measures of C5a were used. First serum levels of C3a des-arg, as a more persistent marker of anaphylatoxin activation. Second, neutrophil surface CD88 levels were measured. Figure IV-9 shows the effect of adding recombinant human C5a (rhC5a) to healthy volunteer neutrophils on the surface expression of CD88, demonstrating a dose-dependent reduction within the range of C5a concentrations reported in sepsis (Solomkin et al. 1981).

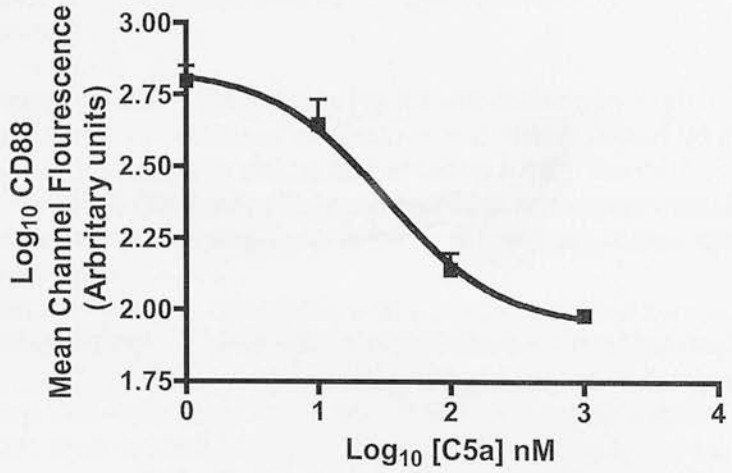


Figure IV-9: Log-Log plot showing the dose response curve of neutrophil CD88 to increasing concentrations of rhC5a
Actual concentrations used were 0,1,10,100 and 1000nM, however 0 point not displayed due to logarithmic axis, geometric mean fluorescence at 0 C5a was 777 (95% CI 607-996). Data shown as mean and standard error of the mean from n=4 healthy donors, r=-0.91, dose at which CD88 is reduced by 50% (EC_{50}) 32nM.

(i) Complement split product C5a as a mediator of impaired phagocytosis – patient data

Assessment of the potential relationship between C5a and phagocytic dysfunction was explored by measuring the correlation between C3a des-arg and phagocytosis of serum opsonised zymosan, as well as the correlation between CD88 and phagocytosis of serum opsonised zymosan. The results of these are shown in figures IV-10 and

IV-12 below. Although there are potential problems with using C5a des-arg as a measure of C5a production (see above), the correlation between C5a des-arg and phagocytosis is also shown below in figure IV-10. High levels of both C5a des-arg and C3a des-arg were found in patient serum (see table III-3, page 109).

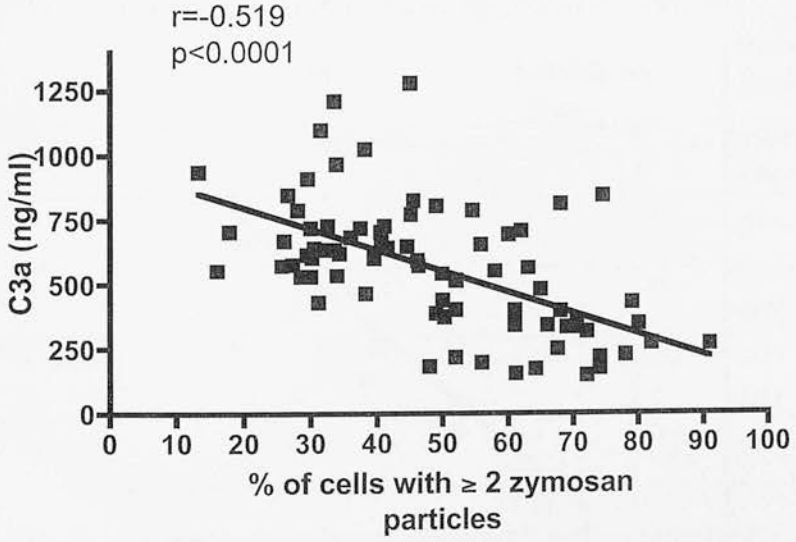


Figure IV-10: Correlation between C3a des-arg and neutrophil phagocytosis.
r and p values by Spearman's rho. Data from n=79 (67 patient and 12 matched volunteer) study participants.

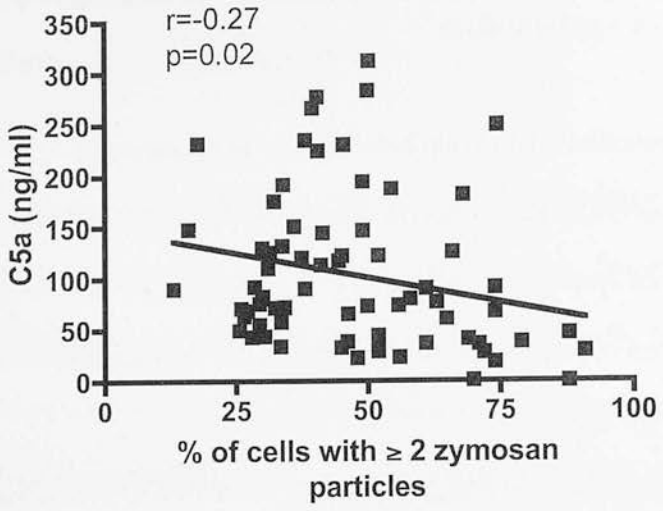


Figure IV-11: Correlation between C5a des-arg and neutrophil phagocytosis.
r and p values by Spearman's rho. Data from n=79 (67 patient and 12 matched volunteer) study participants.

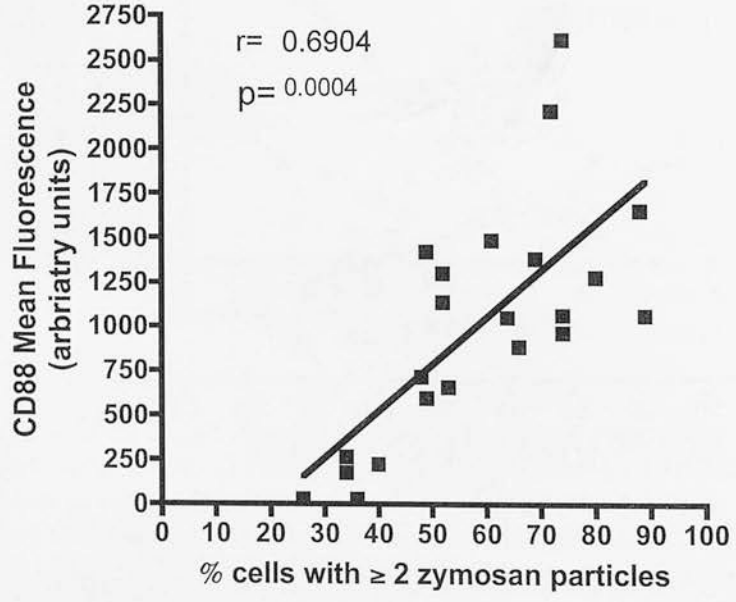


Figure IV-12: correlation between neutrophil surface CD88 expression and phagocytosis.
r and p values by Spearman's rho, data derived from 22 (15 patient and 7 matched volunteer) study participants.

(j) Relationship between phagocytosis and other serum factors associated with inflammation

To examine whether the relationships noted above were specific for complement activation, and C5a in particular, the potential for correlations between other inflammatory molecules (IL-6, IL-8, TNF-α) and the anti-inflamatory cytokine IL-10 and phagocytosis was examined, as shown in table IV-3 no significant correlations were found. Similarly to examine whether the CD88 correlation was specific, or simply a generalised feature of activated neutrophils, the potential for correlations between other markers of neutrophil activation (CD11b, CD16) and the two functional Fcγ receptors (CD32 and 64) and phagocytosis. This data is shown in table IV-4, again there are no significant correlations.

	r (by Spearmans rho)	p (by Spearmans rho)
IL-6	-0.185	0.2
IL-8	-0.01	0.4
TNF-alpha	-0.123	0.3
IL-10	0	0.9

Table IV-4: Correlation co-efficient between cytokine concentration and phagocytosis of zymosan.
r and p values by Spearman's rho, n=79 (67 patient and 12 matched volunteer) study participants for cytokines.

	r (by Spearmans rho)	p (by Spearmans rho)
CD11b	-0.3	0.1
CD16	0.3	0.1
CD32	0.27	0.2
CD64	-0.3	0.1

Table IV-5: Correlation co-efficient between neutrophil phagocytic receptor expression and phagocytosis of zymosan.
Receptor expression defeined by flow cytometric geometric mean fluorescence, r and p values by Spearman's rho, n=29 (21 patient and 8 matched volunteer) study participants for phagocytic receptors.

(k) Complement split product C5a as a mediator of impaired superoxide ion production

As shown in Chapter 3 (figure III-3, page 102), alongside the defect in phagocytosis, neutrophils from patients also produced less superoxide on stimulation with PAF and fMLP. To examine the relationship between C5a and this neutrophil function, which has previously been demonstrated in animal models of sepsis (Huber-Lang et al. 2002b), the correlations between stimulated superoxide production and either C3a des-arg or CD88 were performed (see figures IV-13 and IV-14 below).

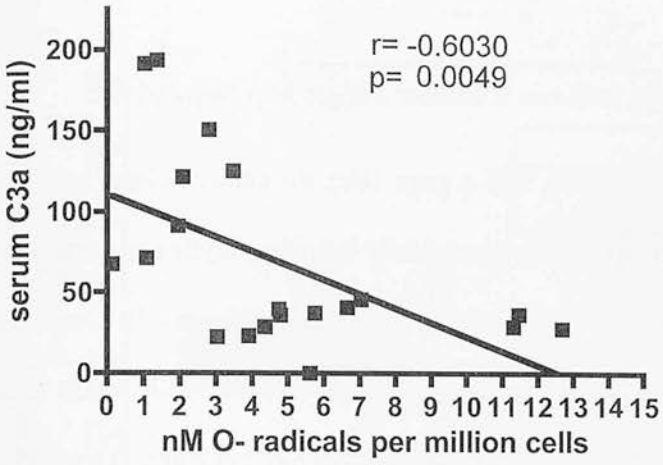


Figure IV-13:Correlation between serum C3a des-arg concentration and PAF/fMLP-stimulated superoxide production.
r and p values by Spearman's rho, data derived from 20 (16 patient and 4 matched volunteer) study participants.

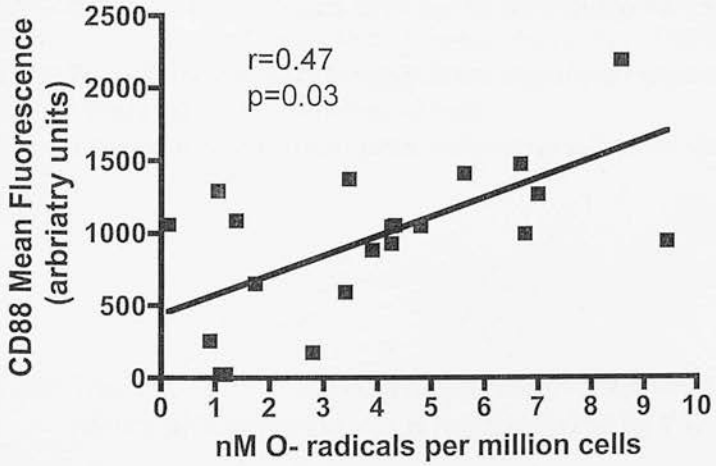


Figure IV-14: Correlation between neutrophil surface CD88 expression and PAF/fMLP-stimulated superoxide production.
r and p values by Spearman's rho, data derived from 20 (16 patient and 4 matched volunteer) study participants.

(I) Patient lavage induces a similar defect in phagocytosis

As noted in Chapter 3 (section 3.02 c, page 100), the cells obtained from the alveolar space of patients displayed markedly less phagocytic capacity than those from healthy volunteers. This was despite a marked difference in composition of those cells, those from patients being predominantly neutrophils whilst those from matched volunteers were almost exclusively macrophages. To explore the defect occurring in the alveolar cells, some of the experiments above were replicated using cell-free lavage supernatant in place of serum. Lavage had no significant opsonising effect on zymosan (data not shown); however incubating healthy volunteer neutrophils with lavage from patients with suspected VAP induced a similar defect in phagocytosis (figure IV-15 below). Notably this effect was not seen when the neutrophils were incubated with lavage from matched volunteers.

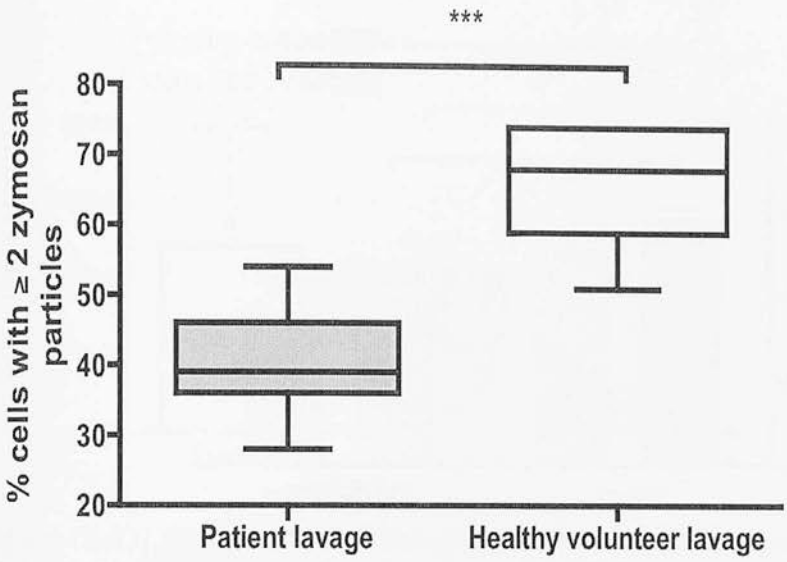


Figure IV-15: The effect of incubating healthy volunteers' blood neutrophils in cell-free bronchoalveolar lavage fluid from patients or matched controls.
Data represent duplicates from 5 separate healthy PBN donors and are expressed as medians, interquartile ranges (box) and range (whiskers), *** p=0.0001 by Mann-Whitney U test.

(m) The observed bronchoalveolar lavage fluid-induced defect in neutrophil phagocytosis is not mediated by C5a

To investigate whether the lavage factor which inhibited phagocytosis was the same as identified in the peripheral circulation (i.e. C5a) the following experiments were conducted.

Healthy donor neutrophils were incubated with either an antibody that prevents C5a binding to CD88 (murine anti-human CD88, clone S5/1) or a control antibody (non-immune murine IgG1). However this was unable to prevent the effect of lavage on phagocytosis (Figure IV-16 below).

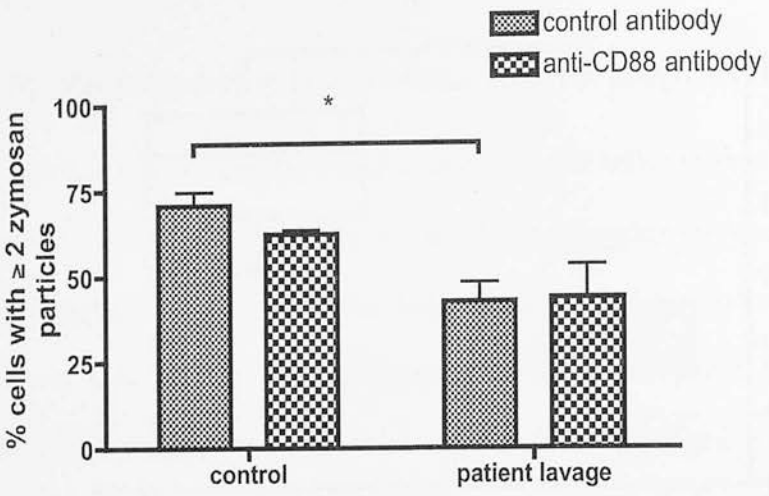


Figure IV-16: The effect of anti-CD88 antibody S5/1 on the ability of patient lavage to induce a defect in phagocytosis
Healthy volunteer neutrophils pre-incubated with S5/1 prior to exposure to patient lavage. Data are shown as median and upper quartile s of duplicates from n=3 separate PBN donors, $p<0.001$ by Kruskal-Wallis, *$p<0.05$ by Dunn's post hoc test.

To determine whether there were significant levels of functional C5a in the BAL fluid from patients, healthy donor neutrophils were incubated with patients' lavage followed by a measurement of surface CD88 expression. As demonstrated above (figure IV-9, page 129) C5a is able to reduce CD88 expression, however lavage produced no significant change (Figure IV-17, below). This is consistent with the comparatively low levels of C3a des-arg and C5a des-arg found in lavage, relative to serum (tables III-3 and III-4, page 109 above).

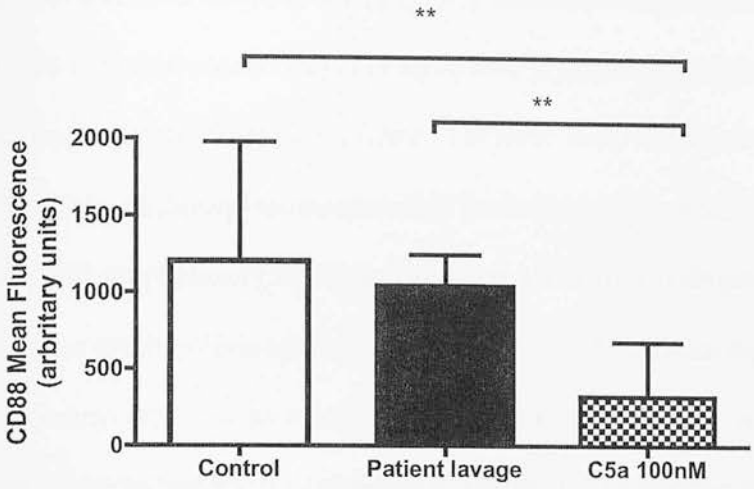


Figure IV-17: Comparison of the effects of patients' lavage or C5a on CD88 expression by healthy blood neutrophils.
Data represent duplicates from 3 separate healthy PBN donors and are expressed as mean and standard deviation, $p<0.0001$ by ANOVA, ***$p<0.001$ by Bonferroni's post hoc test.

Section 4.03 Chapter Summary and Discussion

This work confirms the findings of previous groups that the zymosan ingestion assay used is complement-mediated and acts via CD11b/18 (Allen, Aderem 1996, Le Cabec et al. 2002) resulting in actin polymerisation (Caron, Hall 1998).

Several mechanisms have been proposed to explain findings of impaired phagocytosis in critically ill patients. Defects in opsonisation have been reported in patients at risk of ARDS (Alexander et al. 1978), and serum inhibitors of phagocytosis found in patients with burns (Bjornson, Bjornson & Altemeier 1981). Defects in CD11b expression were found in non-survivors with sepsis (Muller Kobold et al. 2000), whilst Rosenbloom and colleagues showed defects in CD11b activation following stimulation with TNF-α in critically ill patients (Rosenbloom et al. 1999). However it is of note that with the exception of

Bjornson's study, none of the other defects had been linked directly to impairment of phagocytosis.

In this study there was no evidence of defects in serum opsonising ability (Figure IV-6, page 123), or indeed serum inhibitors of phagocytosis (Figure IV-8, page 128). Although total CD11b increased on both patient and volunteer neutrophils on stimulation with zymosan, only the up-regulation on volunteer neutrophils was statistically significant (Table IV-2, page 126). It appears that whilst patient neutrophils show a relative inability to further increase CD11b expression in response to a stimulus, they are able to activate similar amounts of receptor to that achieved by healthy neutrophils. It is also notable that both opsonised and non-opsonised zymosan achieve similar levels of CD11b expression and receptor activation, despite producing markedly different phagocytic responses (Figure IV-1, page 124). These data suggest that, in this patient population at least, the phagocytic defect noted is not due to either deficient CD11b expression or failure of CD11b activation.

In contrast to the other mechanisms explored, this study identified a relationship between markers of C5a exposure and defects in phagocytosis and reactive oxygen species. This is the first time such a relationship has been identified in humans, possibly because of the difficulties in accurately determining the exposure of neutrophils to C5a in 'real-life' rather than experimental situations. This relationship between C5a and neutrophil dysfunction is consistent with animal and *in-vitro* experimental work by Huber-Lang and colleagues (Huber-Lang et al. 2002b)who demonstrated C5a-mediated defects in neutrophil

phagocytosis in a rat caecal ligation and puncture (CLP) model of sepsis as well as C5a-mediated impairment of phagocytosis in healthy human neutrophils exposed to recombinant C5a *in-vitro*. The same study also demonstrated, *in-vitro*, that C5a could impair superoxide production by preventing the translocation of phox47 to the plasma membrane to form part of the NADPH oxidase complex (Huber-Lang et al. 2002a)

It is, however, important to note that at least one other mechanism impairing phagocytosis is occurring in patients. Within the alveolar space a factor, or factors, other than C5a prevents phagocytosis, an effect that appears to be specific to lavage fluid from the lungs of critically ill patients, as it was not induced by lavage from age- and sex-matched volunteers. Although these data suggest that the pulmonary inhibitor is C5a-independent, at present there are no further data on what this inhibitor(s) may be.

The finding of a relationship between C5a and neutrophil dysfunction is both interesting and thought provoking. In contrast to the classical story of a 'counter-balancing' immunosuppressive reaction to inflammation driving dysfunction, in this case it appears to be the pro-inflammatory mediator itself that is directly mediating the defect. However before this postulate can be confirmed, the relationship between C5a and phagocytosis requires further examination to determine whether it is indeed a causative one, and if so what the underlying mechanism may be. It is to this topic that the next chapter turns, to further pursue the hypothesis that C5a is a key mediator of human neutrophil dysfunction in critical illness.

Article V. An examination of the mechanisms by which C5a inhibits phagocytosis.

Section 5.01 Chapter overview and introduction

In chapter 3 the co-existence of hyper-inflammation and immune suppression was demonstrated in ICU patients. Chapter 4 further expanded this 'septic superposition', by showing a relationship between a key pro-inflammatory molecule (C5a) and neutrophil dysfunction. Showing that a single molecule can induce immune cell dysfunction, alongside its more established pro-inflammatory effects which tend to be seen at lower concentrations (Huber-Lang et al. 2002c, Frohlich et al. 1998), provides some mechanistic insights into how the aforementioned state of 'septic superposition' could be achieved.

However, simply showing a relationship between markers of C5a exposure and neutrophil dysfunction is not sufficient to prove causation. Therefore this chapter sets out to explore the mechanism(s) by which C5a is able to induce a defect in phagocytosis, one of the key anti-bacterial functions of the neutrophil.

The hypothesis is that, by ligating its predominant receptor (CD88, not itself a phagocytic receptor), C5a would activate intracellular pathways that would in turn act to inhibit the signalling systems required for phagocytosis and so prevent phagocytosis from happening. C5a, and molecules which ligate receptors coupled to the same G-protein subunits such as fMLP, activate several intracellular pathways (Kelvin et al. 1993). The first pathway explored was the Protein Kinase A (PKA)/adenylate cyclase pathway, as this pathway is activated

by beta-adrenoceptor agonists which also inhibit phagocytosis (Ignarro, Lint & George 1974). When this proved not to be the responsible pathway (see below), a new hypothesis was generated; that activation of PI3K would lead to inhibited phagocytosis.

PI3Ks are a family of key signal transduction molecules, which by catalysing the formation of phosphatidylinositol-trisphosphate, signal a wide range of intracellular pathways (Rommel, Camps & Ji 2007). Highly conserved across species, the PI3K family is divided into 3 sub-families; these are known as type I, II and III, dependent on their structure, regulation and in-vitro substrate specificity (Wymann, Pirola 1998). Type I PI3Ks remain the best studied, are ubiquitous across mammalian cells and catalyse the formation of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3) from phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) (Hirsch et al. 2000). Type I enzymes consist of two components, a larger 110 kDa catalytic sub-unit (designated p110) and a smaller regulatory sub-unit. The regulatory sub-unit further subdivides the type I enzymes into class IA, with a p85 regulator, and class IB with a p101 or p84 regulatory sub-unit. There are three known type IA enzymes, designated PI3Kα, β, and δ respectively, each one indicating a distinct p110 subunit. There is only one known IB enzyme, the PI3Kγ isoform (reviewed in Rommel, Camps & Ji 2007). PI3Kγ and δ are largely confined to immune cells (Rommel, Camps & Ji 2007), and are intimately involved in inflammatory activation (Pinho et al. 2007) that has made them potentially attractive targets for pharmacological modulation.

This chapter will set out a series of experiments designed to explore the mechanism by which C5a inhibits phagocytosis. Starting with confirmation of the ability of recombinant C5a to induce a similar defect in phagocytosis in healthy neutrophils to that seen in patients' neutrophils, there then follows an exploration of the intracellular mechanisms that underpin this effect. Then follows an examination of the potential for treatment, using granulocyte-macrophage colony stimulating factor (GM-CSF), and its potential mechanistic features. The impact of C5a on two other neutrophil functions, namely bactericidal ability against live *Pseudomonas* and transmigration are examined. Finally, having built a mechanistic framework of C5a action, attention returns to neutrophils from critically ill patients to examine the intracellular signalling response to zymosan.

Section 5.02 Results

(a) *In-vitro* C5a exposure can inhibit phagocytosis in healthy neutrophils

Following the finding that phagocytosis in patients correlated with measures of C5a exposure (Figures IV-10-12, pages130-131), experiments were undertaken to determine whether the application of recombinant human C5a (rhC5a) could reproduce the defect in healthy donor neutrophils. Incubation of healthy donor neutrophils with rhC5a could induce a dose-dependent defect in phagocytosis, which could be prevented by pre-incubation with the CD88 blocking antibody S5/1 as demonstrated in figure V-1 below.

Of note incubation with 100nM C5a for 1 hour did not alter the rate of apoptosis or cell necrosis compared to incubation with control (Median % viable cells at 1 hour 93% and 95% respectively, p=0.1 by Mann-Whitney from n=3 healthy

donors), therefore an increase in non-viable cells is unlikely to explain the effects seen, which is consistent with existing data showing C5a exerting an anti-apoptotic effect (Perianayagam et al. 2002)

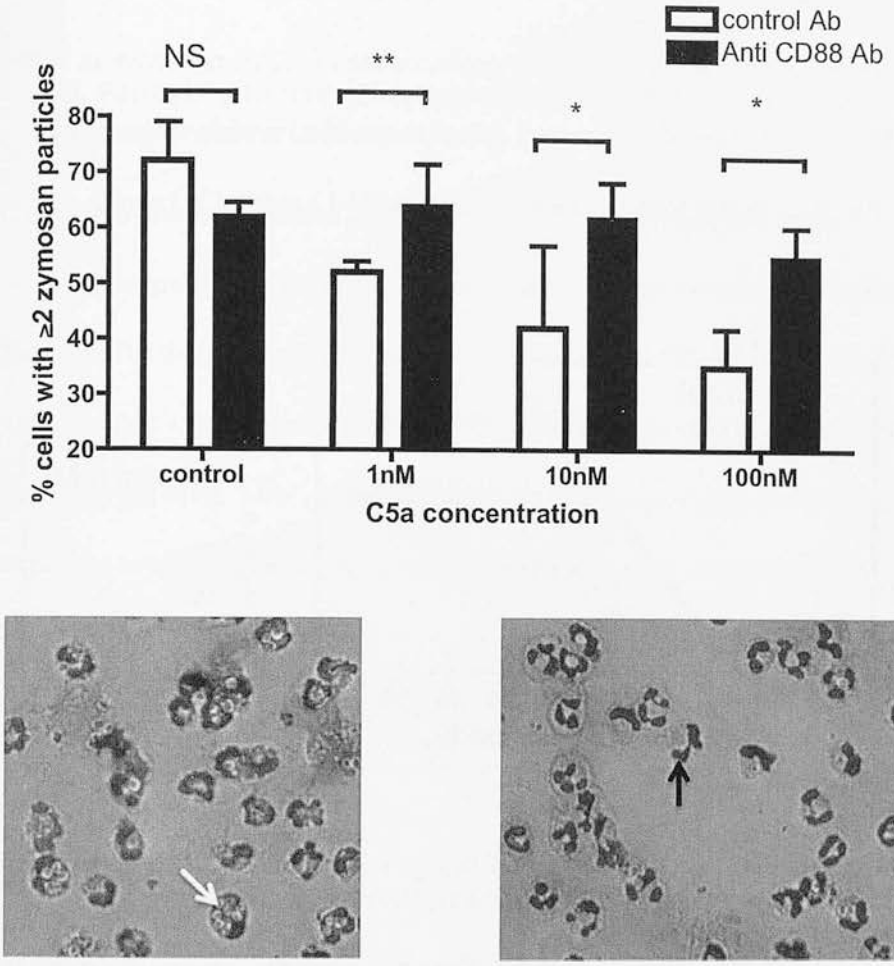


Figure V-1: The effect of recombinant C5a on the phagocytic capacity of healthy human neutrophils in vitro.
Upper graph shows data from neutrophils from healthy volunteers incubated with either S5/1 (a murine monoclonal antibody blocking CD88) or with control murine IgG1antibody prior to C5a exposure. Data derived from duplicates (n=5 PBN donors) and presented as medians and upper quartile.
$p<0.0001$ by Kruskal-Wallis, *$p<0.05$ **$p<0.01$ by Dunn's post hoc test.
Lower photomicrographs left panel - control-treated neutrophils, white arrow indicates a cell which has ingested multiple zymosan particles (the grey circles with a clear rim around each; right panel - rhC5a-treated neutrophils, black arrow indicates a cell which has not ingested zymosan (magnification X32).

Furthermore, incubation with rhC5a produced the expected reduction in surface CD88 expression and demonstrated a similar correlation with phagocytosis (Figure IV-2) to that seen in patients in Figure IV-12 (page 131) above.

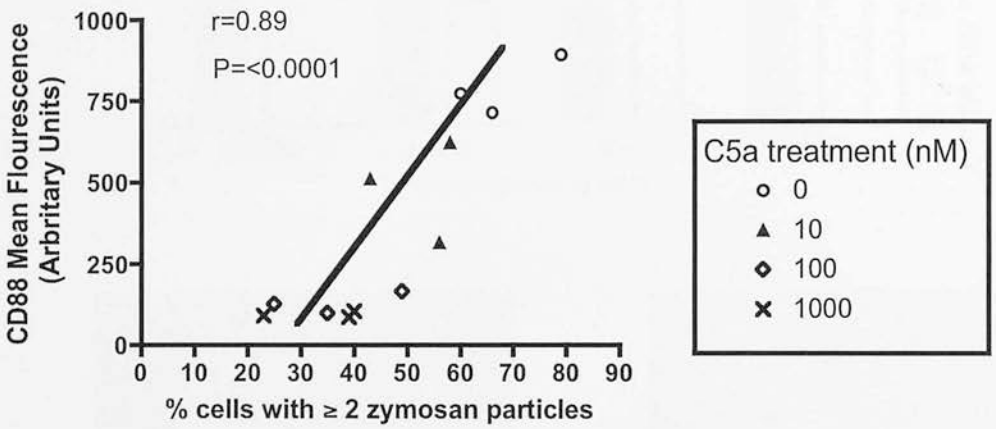


Figure V-2: Correlation between phagocytosis and CD88 expression in healthy volunteer neutrophils exposed to escalating concentrations of C5a

n=3 PBN donors. Three separate donors' neutrophils were exposed to 4 separate doses of C5a, i.e. each symbol x is from a different donor's neutrophils exposed to 1000nM C5a etc. r and p values by Spearman's Rho.

(b) Formyl methionyl leucyl phenylalanine (fMLP) and C5a induce a similar defect in phagocytosis.

To explore the mechanisms underlying the defect in phagocytosis, the capability of other neutrophil activators to induce defects in phagocytosis was examined. Specifically fMLP was examined, as its receptor (fMLPR) is coupled to the same $G_{\alpha i}$subunit as CD88 (Skokowa et al. 2005, Bommakanti et al. 1995) and hence activates the same intracellular pathways. In equimolar concentrations fMLP was able to inhibit phagocytosis in a manner similar to C5a (figure V-3).

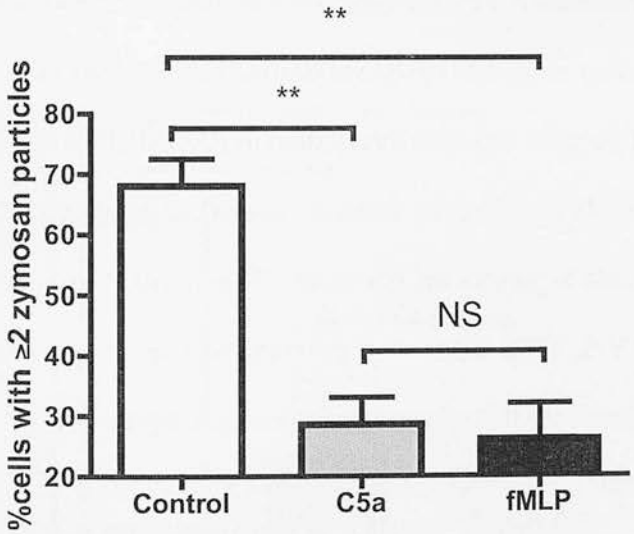


Figure V-3: The effects of C5a and fMLP on phagocytosis by healthy neutrophils.
Data are presented as median and inter-quartile ranges, $p<0.0001$ by Kruskal-Wallis, NS $p>0.05$, **$p<0.01$ by Dunn's post-hoc test, (n=6 individual PBN donors, all experiments performed in duplicate).

(c) cAMP mediates beta-adrenoceptor agonist-induced defects in phagocytosis but not those of C5a and fMLP.

One downstream pathway of fMLP and C5a involves PKA and adenylate cyclase activation, resulting in increased levels of cyclic adenosine monophosphate (cAMP). As beta-adrenoceptor agonists are reported to impair phagocytosis via activation of cAMP (Ignarro, Lint & George 1974), the effect of isoproterenol on the phagocytosis assay was assessed. Neutrophils incubated with 100μM isoproterenol (a concentration used in previous studies (Ignarro, Lint & George 1974)) showed impaired phagocytosis, an effect which could be blocked by pre-incubating the cells with the adenylate cyclase inhibitor SQ22536 (Figure V-4). A similar effect could be achieved by activation of adenylate cyclase by an alternative ligand, pituitary adenylate cyclase activating peptide (PACAP) (phagocytosis reduced from 74% to 45% by addition of PACAP, P=0.002 by Mann-Whitney, results from n=5 healthy donors). In marked contrast SQ22536 pre-incubation was unable to prevent the effects of C5a and fMLP on phagocytosis (Figures V-5, V-6). These divergent results could be explained by the differential response of cAMP to the various stimulants, figure V-7 demonstrating that cAMP is transiently elevated after C5a and fMLP, at concentrations of these agonists which inhibit phagocytosis, but experiences more prolonged and increasing elevation with isoproterenol.

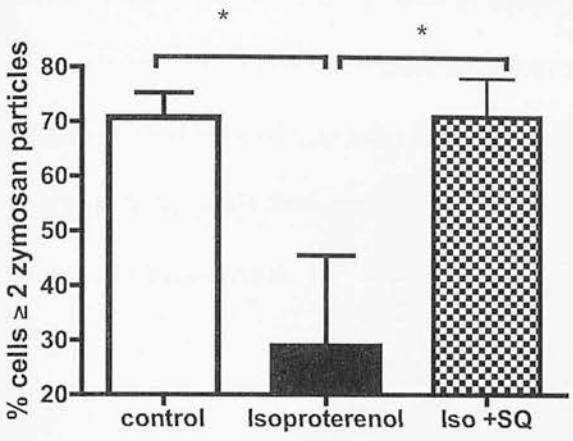


Figure V-4: Phagocytosis by healthy donor PBNs exposed to the beta-adrenoceptor agonist isoproterenol with or without pre-incubation with the adenylate cyclase inhibitor SQ22536.
p=0.006 by Kruskal-Wallis, *p<0.05 by Dunn's post-hoc test (n=5 individual PBN donors, all assays performed in duplicate).

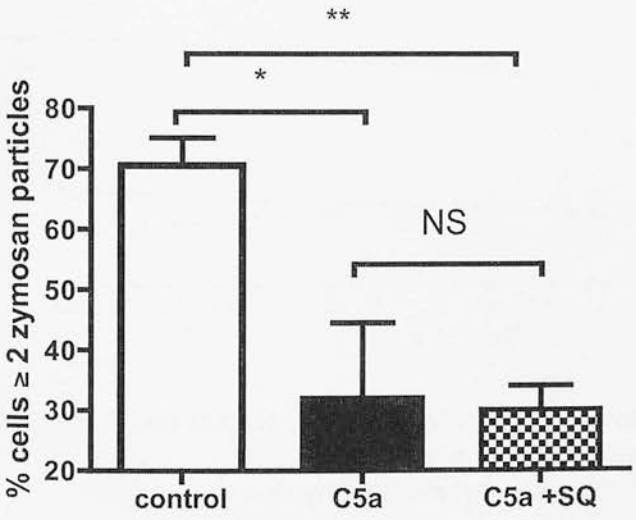


Figure V-5: Phagocytosis by healthy donor PBNs exposed to 100nM C5a with or without pre-incubation with SQ22536.
p=0.003 by Kruskal-Wallis, *p<0.05, **p<0.01 by Dunn's post-hoc test (n=5 individual PBN donors, all assays performed in duplicate).

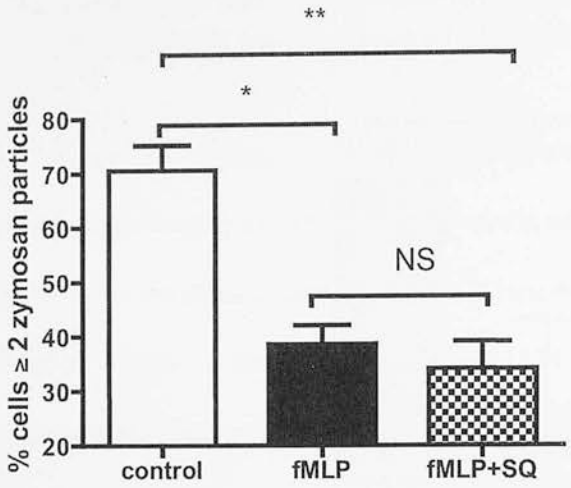


Figure V-6: Phagocytosis by healthy donor PBNs exposed to 100nM fMLP with or without pre-incubation with SQ22536.
p=0.003 by Kruskal-Wallis, *p<0.05, **p<0.01 by Dunn's post-hoc test (n=5 individual PBN donors, all assays performed in duplicate).

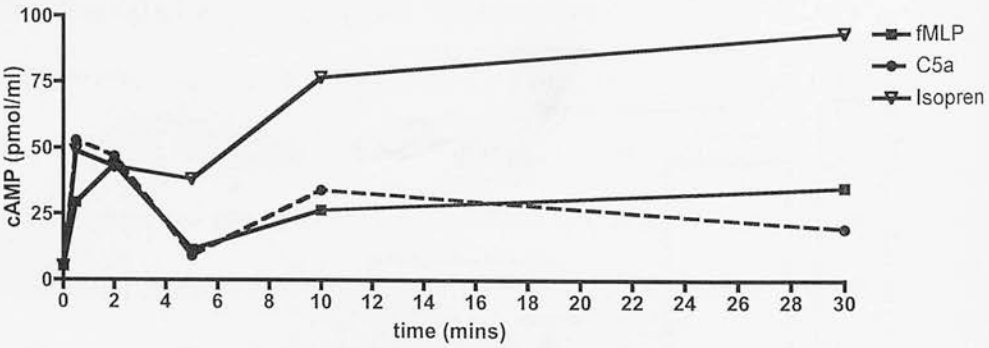


Figure V-7: effect of three stimuli (100nM C5a, 100nM fMLP, 100μM isoproterenol) on cAMP levels in neutrophils.
Data shown as median values from n=4 healthy donors.

(d) C5a acts via PI3K to inhibit phagocytosis

Following the ruling out of cAMP as the mediator of C5a- (and fMLP-) induced defects in phagocytosis, attention turned to other intracellular mechanisms linked to actin polymerisation and phagocytosis. The phosphoinositide 3-kinase family have been implicated in a wide range of signalling pathways, however within

leucocytes they are known to affect the small Rho GTPases that are key mediators of actin polymerisation, such as occurs during phagocytosis (Papakonstanti et al. 2008). Therefore the effect of inhibiting class I PI3K was examined using the broad-spectrum PI3K inhibitors wortmannin and LY294002 (figure V-8, V-9). This demonstrated that C5a required PI3K to effect its inhibition of phagocytosis.

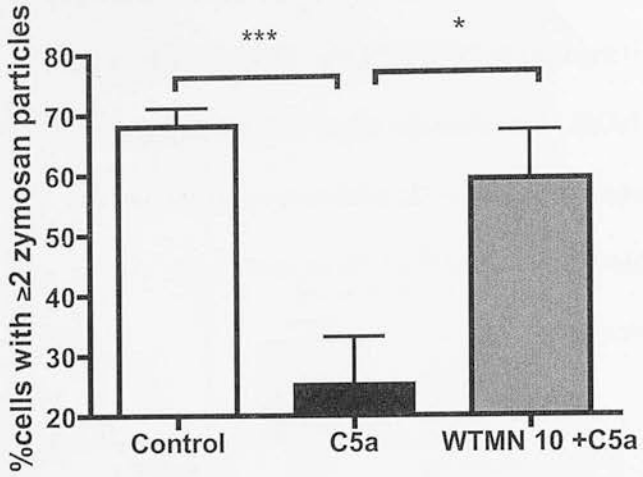


Figure V-8: Phagocytosis by healthy donor PBNs exposed to 100nM C5a with or without pre-incubation with 10nM wortmannin.
P=0.0003 by Kruskal-Wallis, *p<0.05, ***p<0.001 by Dunn's post-hoc test (n=5 individual PBN donors, all assays performed in duplicate).

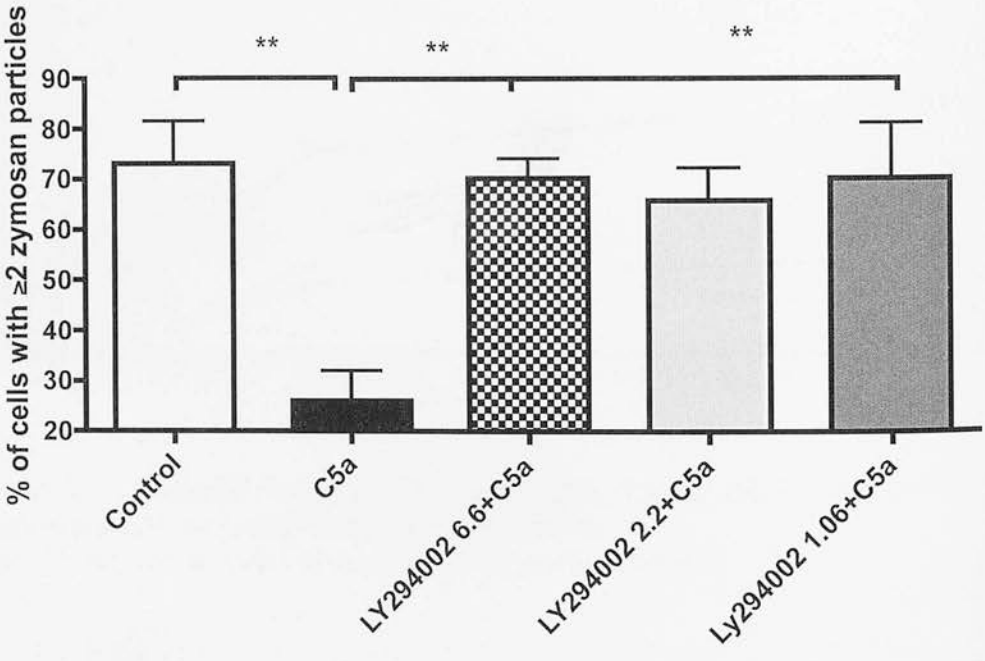


Figure V-9: The effect of pre-incubation of healthy neutrophils with varying concentrations of LY294002 before exposure to 100nM C5a.
6.6μM being IC_{50} for PI3Kγ Data are illustrated as median and inter-quartile range, p=0.0002 by Kruskal-Wallis, **p<0.01 by Dunn's post-hoc test (n=6 individual PBN donors, all experiments performed in duplicate).

The effects of fMLP on phagocytosis were similarly affected by wortmannin (control 68%, fMLP 20%, wortmannin +fMLP 70%, P=0.003 by Kruskal-Wallis n=5 donors) and LY294002 (control 68%, fMLP 22%, LY294002+fMLP 74%, P<0.001 by Kruskal-Wallis n=5 donors).

At the concentrations used, the PI3K inhibitors did not themselves affect phagocytosis. However in dose ranging experiements concentrations of LY294002 over 26μM started to inhibit phagocytosis, and at the IC_{90} of PI3Kγ - 59μM – phagocytosis fell from 70% to 12% (n=2), consistent with the involvement of PI3K in phagocytosis. Interestingly the ability of LY294002 to prevent C5-induced defects in phagocytosis persisted as concentrations dropped below 6.6μM dose, the lowest dose examined being 1.06 μM which is below the calculated IC_{15} for PI3Kγ, but is the IC_{50} for another class I PI3K isoform, PI3Kδ. However, although there is a degree of dose-related specificity of LY294002, the IC_{50}s are too close to be sure that such a finding reflects a genuine isoform-specific effect (Ferrandi et al. 2007).

(e) C5a inhibits phagocytosis via PI3Kδ and not PI3Kγ

Both wortmannin and LY294002 have been found to inhibit enzymes other than PI3K (Davies et al. 2000), and as noted above have relatively close values for the IC_{50} of the major class I PI3K isoforms (Ferrandi et al. 2007). To further explore the relative roles of the isoforms PI3Kγ and PI3kδ, inhibitors with greater specificity were used. AS605240 has an IC_{50} of 8nM for PI3Kγ compared to 60nM for PI3kδ (Smith et al. 2007), whereas IC87114 has an IC_{50} 0.18μM for

PI3Kδ compared to 23μM for PI3Kγ (Knight et al. 2006) . The effects of these two compounds on C5a-induced defects in phagocytosis were examined (Figure V-10, V-11 A). A further investigation of the specificity and dose response of IC87114 is presented in figure V-11 B, demonstrating that increasing the dose to the IC_{50} of PI3Kγ had no additional benefit on inhibiting C5a's effect on phagocytosis. Furthermore the effect of IC87114 persisted down to 60nM, a concentration over 100-fold lower than that required to inhibit PI3Kγ.

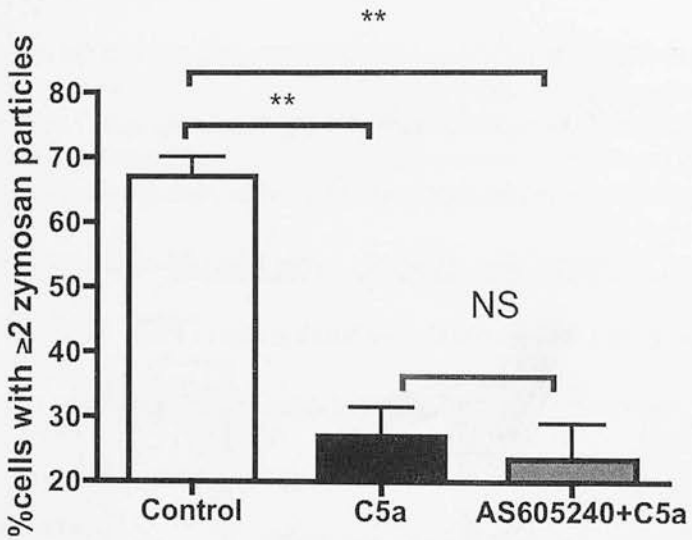


Figure V-10: The effect of rhC5a on phagocytosis is not prevented by pre-incubation with PI3Kγ inhibitor AS605240 at 8nM.
P=0.02 by Kruskal Wallis ANOVA, *p<0.05, **p<0.01 by Dunn's post-hoc test. Data are shown as median and inter-quartile range (n=5 healthy volunteers, all experiments performed in duplicate).

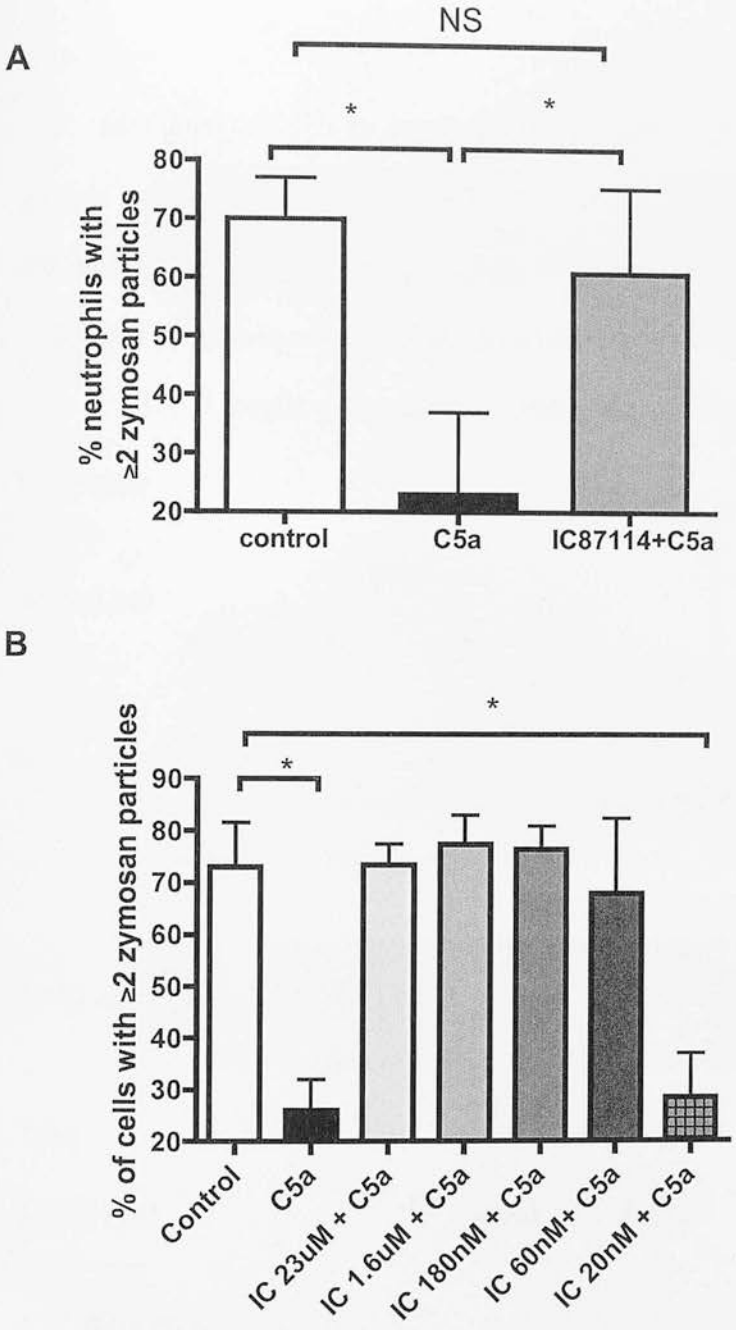


Figure V-11: The effect of rhC5a on phagocytosis is prevented by pre-incubation with the PI3Kδ inhibitor IC87114.
Panel A: **IC87114 at 180nM (IC_{50} for PI3Kδ)**. P=0.008 by Kruskal-Wallis ANOVA, *p<0.05 by Dunn's post-hoc test. Data are shown as median and inter-quartile range (n=5 healthy volunteers, all experiments performed in duplicate).
Panel B: **Various concentrations of IC87114.** Concentrations represent the IC_{50} of PI3Kγ (23μM) and $IC_{90,\ 50,\ 25}$ and $_{10}$ of PI3Kδ. P=0.0006 by Kruskal-Wallis, *P<0.05 by Dunn's post-hoc test. (n=6 healthy volunteers, all experiments performed in duplicate).

To confirm that the AS605240 was functional, its ability to inhibit the phosphorylation of protein kinase B (PKB) was assessed by western blotting (figure V-12A). Further assessment of the specificity of IC87114 for the delta isoform was demonstrated by showing a failure to completely inhibit PKB phosphorylation at doses that inhibited phagocytosis (figure V-12B).

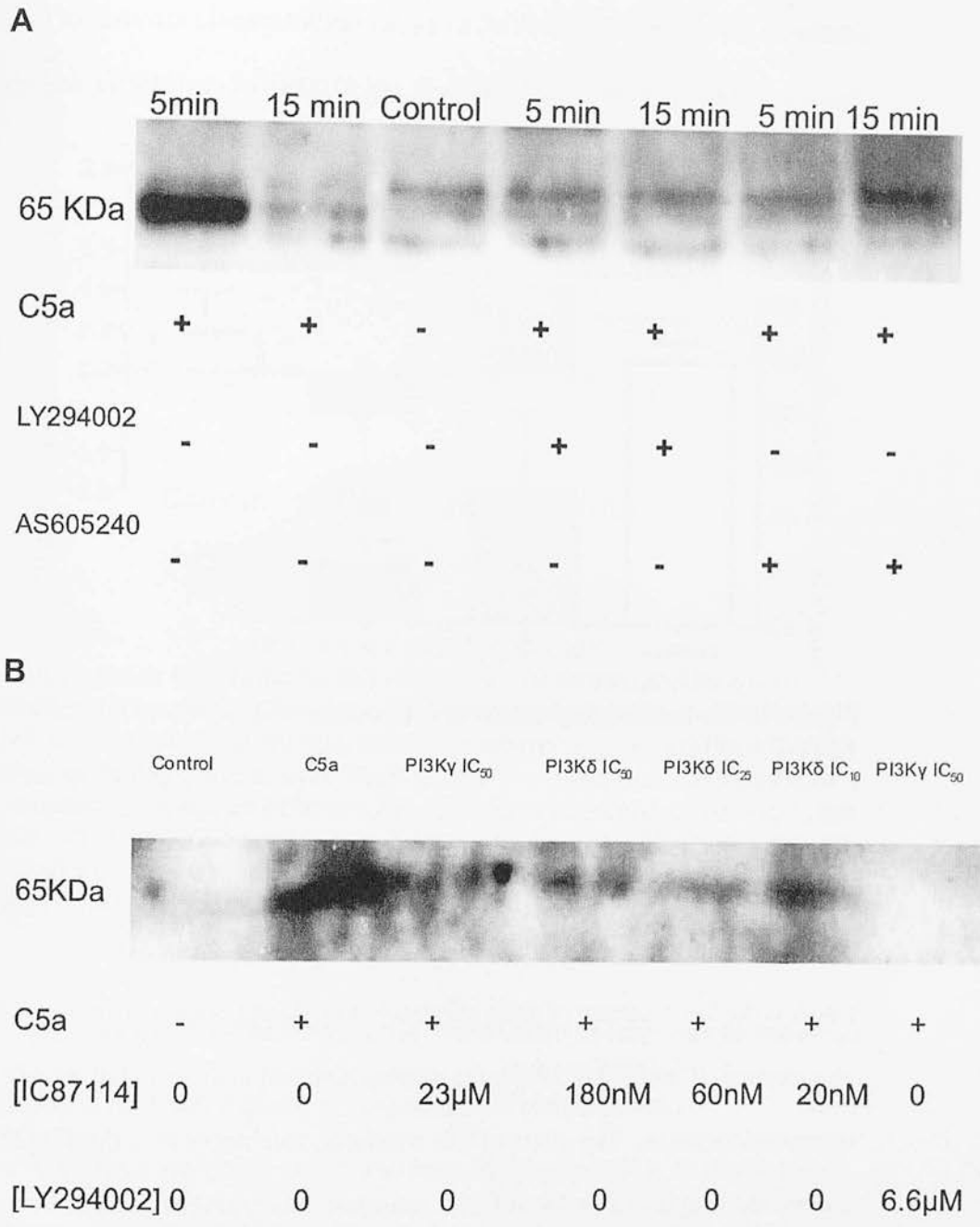


Figure V-12: Western blots showing phospho-PKB following stimulation with C5a.
Panel A shows the effects with and without pan-PI3K (LY294002) and PI3Kγ (AS605240) inhibitors. Representative example of n=3 experiements.
Panel B shows the effects of PI3Kδ inhibitor IC87114 at varying concentrations at 5 minutes.

Exposure of neutrophils to 740Y-P, a cell permeable specific activator of the p85 subunit of class IA PI3Ks (i.e. PI3K α, β, and δ) (Derossi et al. 1998), was able to reproduce the defect in phagocytosis (Figure V-13).

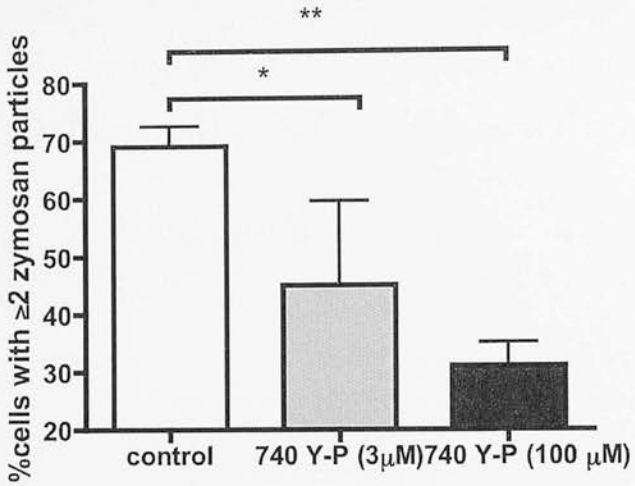


Figure V-13: Incubating neutrophils with the PI3KIA (α,β,δ) activator 740Y-P inhibits phagocytosis.
P=0.01 by Kruskal-Wallis ANOVA, *p<0.05, **p<0.01 by Dunn's post-hoc test. Data are shown as median and inter-quartile range (n=5 healthy volunteers).

(f) C5a activation of PI3Kδ inhibits RhoA activation

RhoA is the key mediator of actin polymerisation during complement-mediated phagocytosis (Caron, Hall 1998), the process involved in ingestion of serum-opsonised zymosan. The effect of C5a treatment, and blockade of this by PI3Kδ inhibition, on zymosan-induced RhoA activation was examined. C5a prevented the activation of RhoA seen in control-treated neutrophils, and this inhibitory effect was dependent on the activation of PI3Kδ (Figure V-14). Alternative Rho GTPases (RAC and CDC42) were not inhibited by C5a (median change in RAC 0.13 pg/ml in control vs. 0.16pg/ml in C5a-treated cells, p=1 by Mann Whitney. Median change in CDC42 -0.01 pg/ml in control vs.-0.02 pg/ml in C5a-treated cells, p=1 by Mann Whitney).

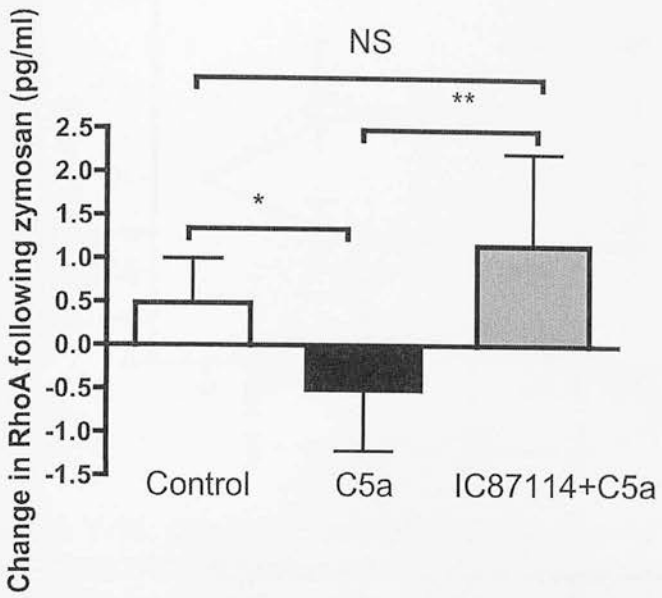


Figure V-14: C5a inhibits the activation of neutrophil RhoA in response to opsonized zymosan, and pre-incubation with PI3Kδ inhibitor IC87114 prevents this inhibition.
Concentrations of reagents are the same as in the phagocytosis experiements shown in 5-11A (i.e. 100nM for C5a and 180nM for IC87114). P=0.005 by Kruskal-Wallis ANOVA, *p<0.05, **p<0.01 by Dunn's post-hoc test. Data presented as median and inter-quartile ranges (n=10 healthy donors).

(g) C5a inhibits the polymerisation of actin in response to zymosan.

Actin polymerisation allows the engulfment of phagocytic targets, and paralysis of this system inhibits phagocytosis (May, Machesky 2001). As shown above (Figure IV-4, page 121) the polymerisation and depolymerisation of actin followed the same time course as the 'velocity' of phagocytosis in our model. Treatment of healthy volunteer neutrophils with C5a inhibited this polymerisation response (figure V-15). Indeed there was a small reduction in filamentous actin (f-actin) within neutrophils, consistent with the reduction in active RhoA noted in Figure V-14. Pre-treatment with the PI3Kδ inhibitor IC87114 prevented this effect (hatched line in figure V-15), again mirroring the

changes in RhoA activation. A similar effect was achieved with the pan PI3K inhibitor LY294002 (figure V-16).

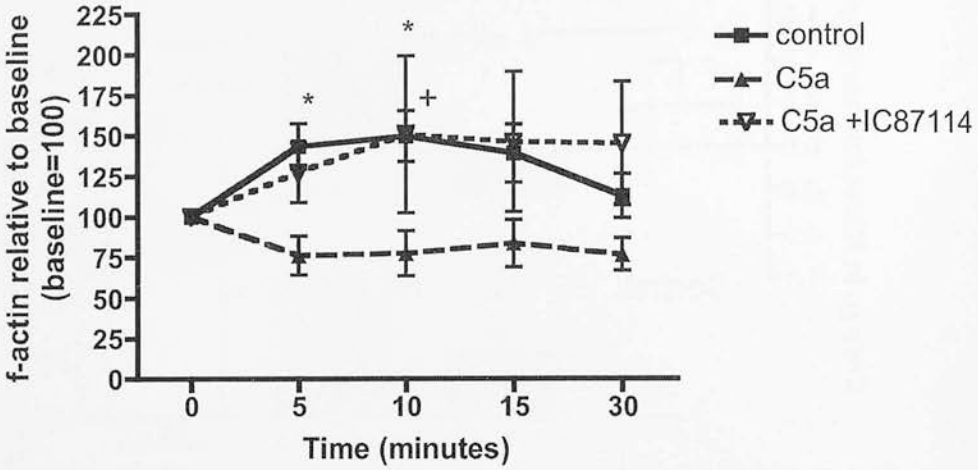


Figure V-15: Change in polymerised (f) actin following zymosan exposure with and without IC87114.
Data from time series in neutrophils treated with vehicle control (squares+solid line), rhC5a (triangles +hashed line) or IC87114 followed by rhC5a (circles +hashed line), then exposed to opsonised zymosan. Data shown as mean and standard error of the mean (SEM), $p<0.0001$ for difference between treatment groups by two way ANOVA, $^{*}p<0.05$ between control and C5a treatment, $^{+}$ $P<0.05$ between C5a+IC87114 and C5a treatment by Bonferroni's post-hoc test (n=8 healthy donors).

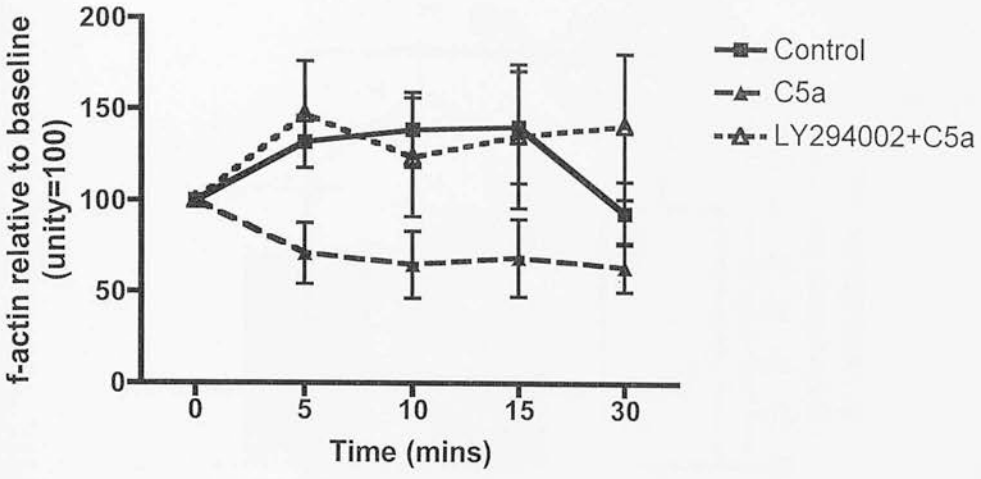


Figure V-16: Change in polymerised (f) actin following zymosan exposure with and without LY294002.
Data from time series in neutrophils treated with vehicle control (squares+solid line), rhC5a (triangles +hashed line) or LY294002 followed by rhC5a (open triangles +hashed line), then exposed to opsonised zymosan. Data shown as mean and standard error of the mean (SEM), p=0.0007 for difference between treatment groups by two way ANOVA, (n=5 healthy donors).

(h) GM-CSF can rescue phagocytosis after C5a exposure.

Ex-vivo application of GM-CSF to healthy donor neutrophils previously exposed to 100nM C5a reversed the defect in phagocytosis (Figure V-17 below). In keeping with the pathway explored above, GM-CSF treatment was associated with a restoration of RhoA activation and actin polymerisation in response to zymosan (Figures V-18 and V-19 respectively).

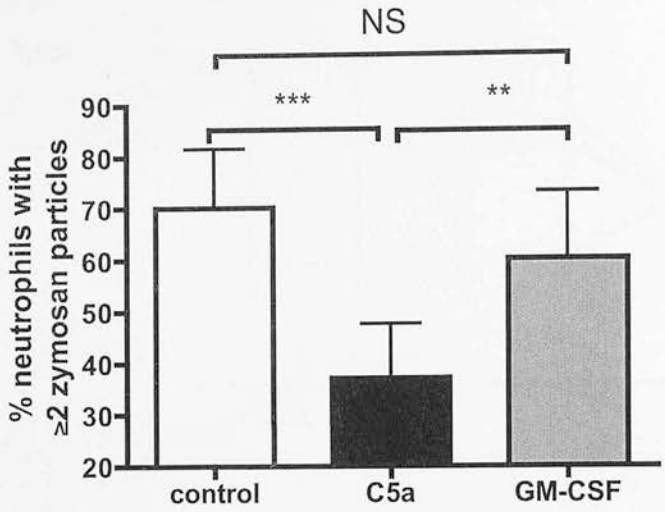


Figure V-17: Addition of GM-CSF restores effective phagocytosis of opsonized zymosan in neutrophils exposed to C5a.
P<0.0001 by Kruskal-Wallis, **p<0.01, ***p<0.001 by Dunn's post hoc test. Data are displayed as median and inter-quartile range (n=10 healthy donors).

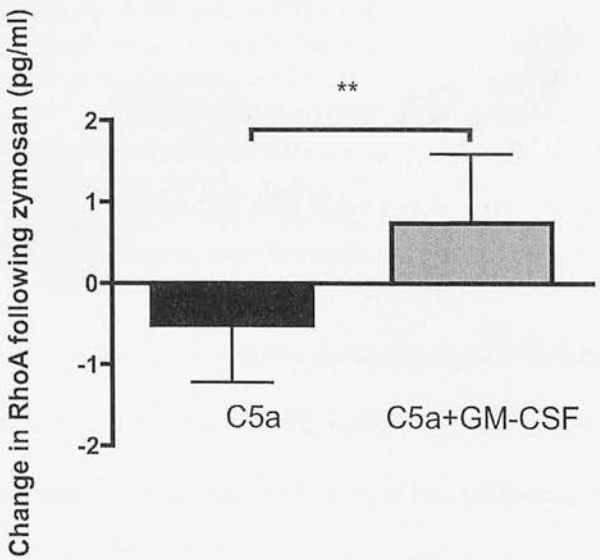


Figure V-18: Addition of GM-CSF restores RhoA activation in neutrophils treated with rhC5a and exposed to opsonised zymosan.
**P=0.007 by Mann-Whitney U-test. Data are displayed as median and inter-quartile range (n=10 healthy donors). Note: C5a data are the same as presented in Figure V-14 above, the GM-CSF data are shown separately for reasons of clarity – separation does not alter the statistical significance of the differences noted.

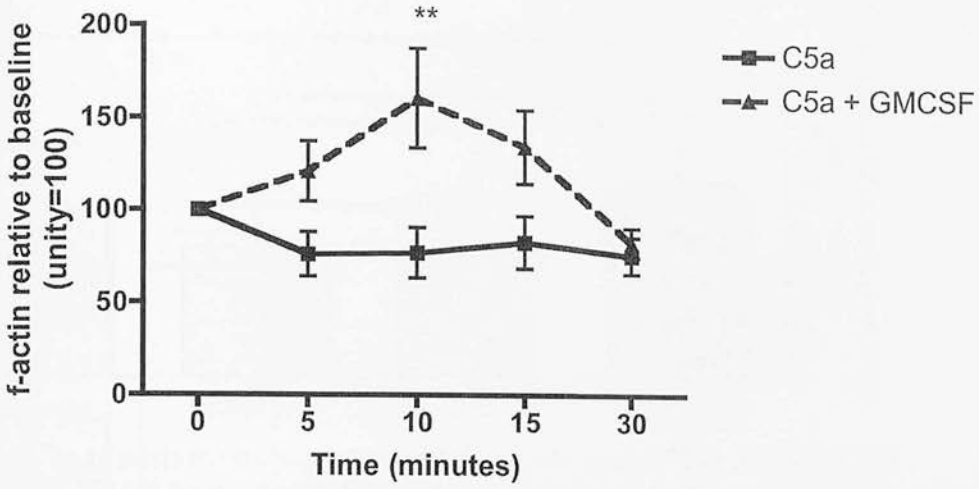


Figure V-19: Addition of GM-CSF restores actin polymerisation in neutrophils treated with rhC5a and exposed to opsonised zymosan. P=0.0001 for differences between treatments by two-way ANOVA, ** p<0.01 by Bonferroni's post-hoc test. Data are shown as mean and SEM (n=8 healthy donors).

(i) GM-CSF does not exert its effect via restoration of CD88 expression or PI3K-delta inhibition.

To explore the potential mechanism by which GM-CSF is able to restore phagocytosis, two avenues were explored. First, given the correlation between CD88 and phagocytosis, the effect on CD88 expression was examined. The addition of GM-CSF to C5a-treated cells did not restore CD88 expression (V-20). In contrast recombinant human interferon γ that does increase CD88 expression (Burg et al. 1995) and figure V-20) was unable to restore phagocytosis (figure V-21).

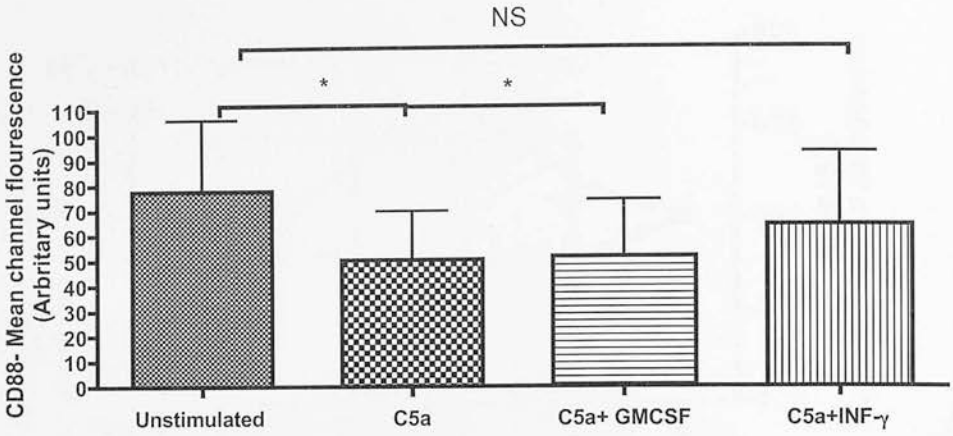


Figure V-20: CD88 expression following ex-vivo treatment of neutrophils with 100nM C5a, followed by addition of 0.3nM GM-CSF or 0.3nM INFγ.

p=0.01 by ANOVA, * p<0.05 by Bonferroni's post-hoc test. n=5 individual healthy donors.

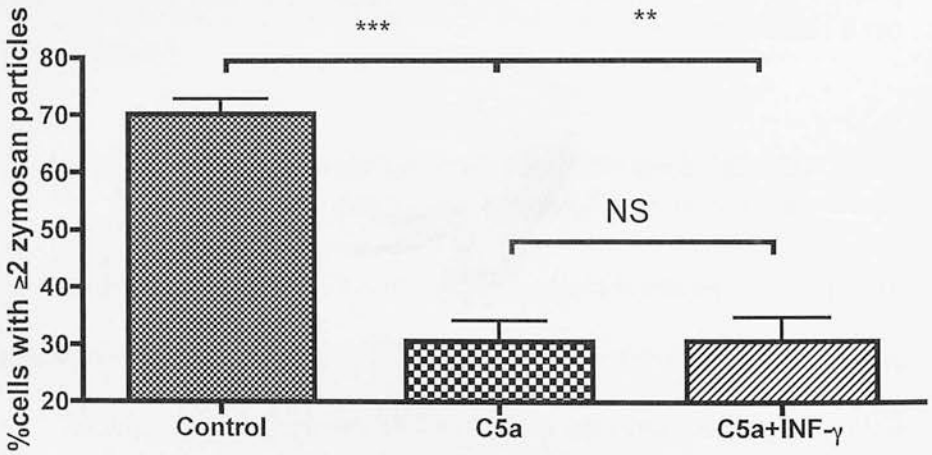


Figure V-21. Phagocytosis by neutrophils exposed to 100nM C5a, followed by 0.3nM IFNg or control.

p=0.009 by Kruskal-Wallis, ** p<0.01, ***p<0.001 by Dunn's post-hoc test.

Second, given the effect of inhibiting PI3K and PI3K delta prior to C5a exposure on preventing phagocytosis, the ability of PI3K delta inhibition by IC87114 to rescue phagocytosis was examined (Figure V-22). Unlike GM-CSF, PI3Kdelta inhibition was unable to rescue phagocytosis suggesting that GM-CSF's effects are independent of this mechanism of action.

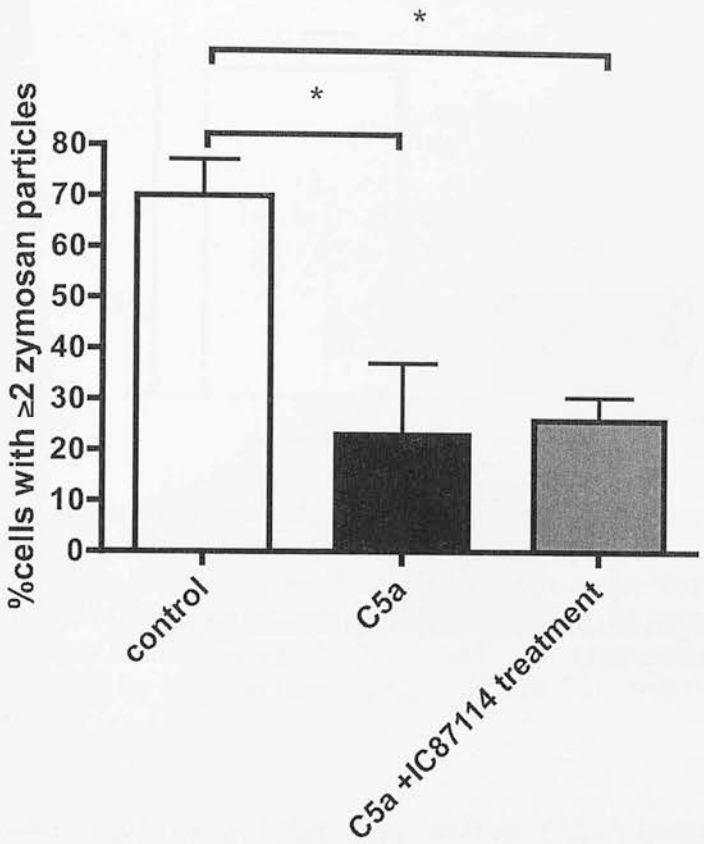


Figure V-22: Addition of PI3Kδ inhibitor IC87114 after 30 minutes treatment with C5a fails to resurrect phagocytosis.
P=0.009 by Kruskal-Wallis, *P<0.05 by Dunn's post-hoc test. Data are displayed as median and inter-quartile range (n=5 healthy donors).

(j) C5a impairs bactericidal capacity of neutrophils

Phagocytosis of zymosan involves the ingestion of large (3-4 micron) particles, which stimulate toll-like receptor 2 (TLR-2) (Dillon et al. 2006). Furthermore ingestion does not necessarily equate to killing (Garcia-Rodas et al. 2011). These issues were examined by investigating the effects of C5a on neutrophil killing of *Pseudomonas aeruginosa,* a common bacterial cause of nosocomial

infections (Peleg, Hooper 2010) that stimulates TLR-4 (Dehus, Hartung & Hermann 2006) in contrast to zymosan which stimulates TLR2.

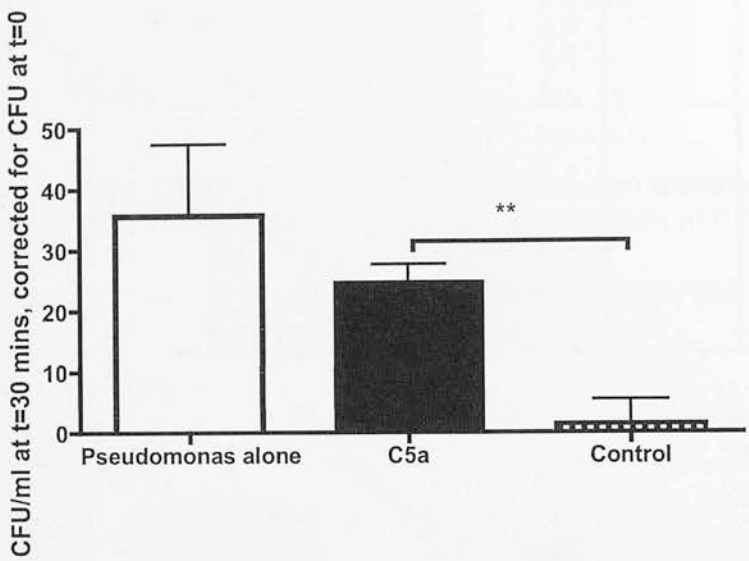


Figure V-23: Effect of C5a on the bactericidal capacity of neutrophils.
P. aeruginosa strain PA01 was added for 30 minutes to wells containing no cells (left column), or to healthy neutrophils pre-incubated with either 100nM C5a (middle column) or control medium (right column). Residual bacteria were quantified and expressed as colony forming units (CFU) above those at t_0.
** p=0.003 by Mann-Whitney (duplicates of n=5 healthy PBN donors).

(k) C5a impairs transmigration of neutrophils

The effect of C5a on actin polymerisation, coupled with reports of transmigratory failure in sepsis (Ahmed et al. 1999) prompted an examination of the effects of C5a treatment on transmigration towards a chemotactic target (Figure V-24). This demonstrated that pre-incubation with C5a was able to prevent transmigration towards fMLP.

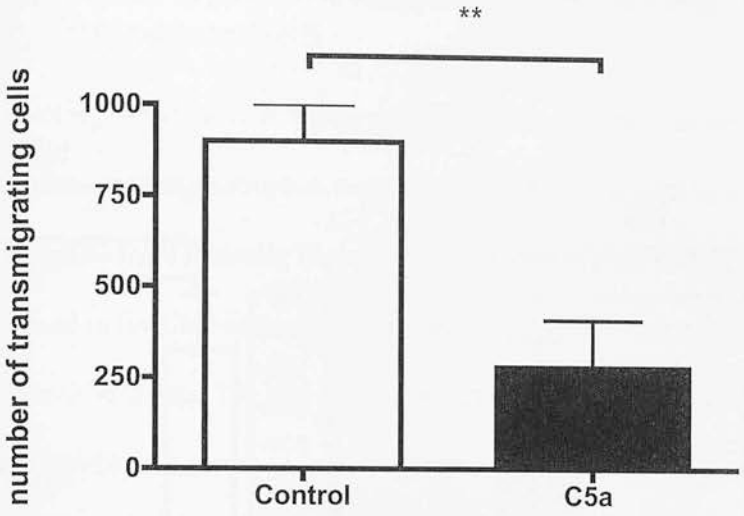


Figure V-24: Transmigration of neutrophils across a 3 micron pore polystyrene membrane to a target of 100nM fMLP.
Neutrophils from healthy volunteers were pre-incubated in control media or 100nM C5a, treated cells applied at 100,000 per insert and incubated for 1 hour at 37°C.
** p=0.008 by Mann-Whiney test (duplicates from n=5 healthy PBN donors).

To examine whether PI3Kδ is central to the impairment of transmigration induced by C5a, the effects of the inhibitor (IC87114) and activator (740Y-P) on transmigration were assessed. In contrast to phagocytosis, IC87114 alone inhibited transmigration and 740 Y-P had no inhibitory effect (Figure V-25A). Unsurprisingly IC87114 was unable to prevent the defect in transmigration induced by C5a, however treatment with GM-CSF was (Figure V-25B), again pointing to GM-CSF operating via PI3Kδ-independent pathways.

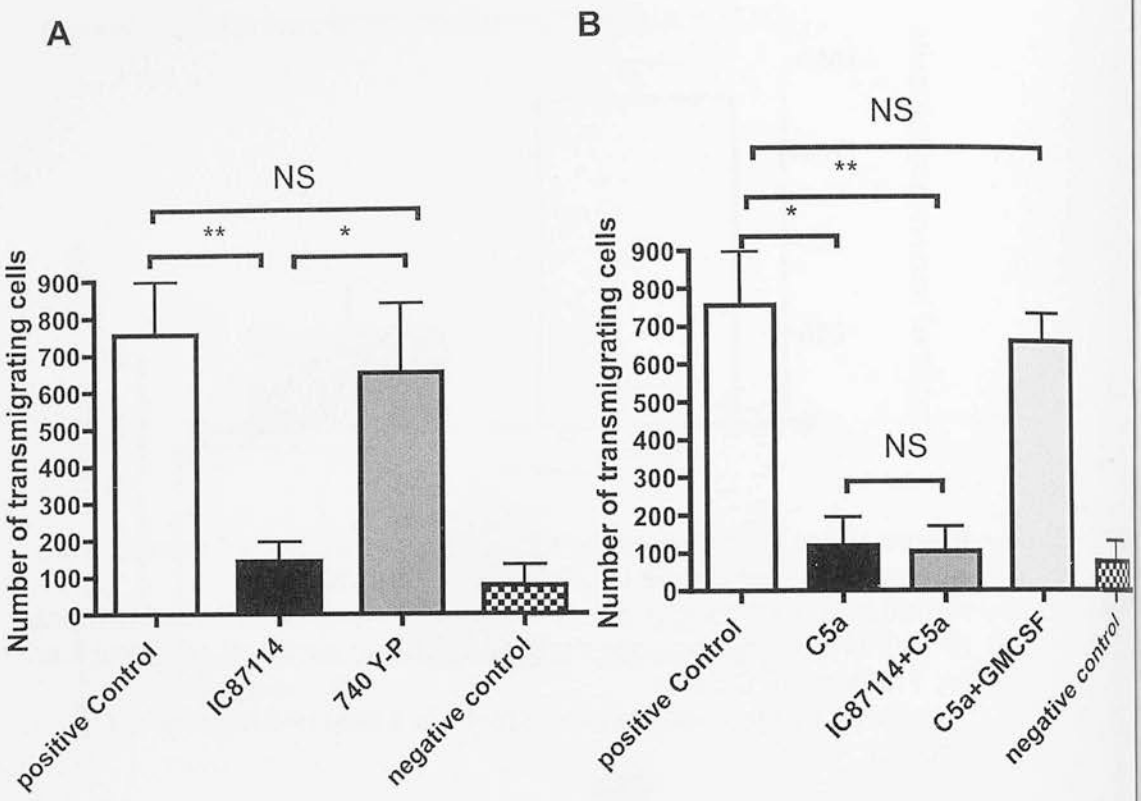


Figure V-25: The effects of C5a and PI3Kdelta modulation on neutrophil transmigration.

Panel A: **transmigration by neutrophils across a 3micron transwell membrane towards an fMLP target following pre-treatment with vehicle control, 180nM IC87114 or 30µM 740 Y-P**. Negative control is without fMLP. p=0.0029 by Kruskal-Wallis, *p<0.05, **p<0.01, NS p>0.05 by Dunn's post-hoc test.

Panel B **shows transmigration by neutrophils across a 3micron transwell membrane towards an fMLP target following pre-treatment with vehicle control, 100nM C5a, 180nM IC87114 followed by 100nM C5a or 100nM C5a followed by 0.3nM GM-CSF.** Negative control is without fMLP. p=0.0005 by Kruskal-Wallis, *p<0.05, **p<0.01, NS p>0.05 by Dunn's post-hoc test.

(I) Defects in patient neutrophil phagocytosis mirror those seen in rhC5a-treated cells

Dissecting out the way in which rhC5a was able to inhibit zymosan phagocytosis in healthy human neutrophils suggested a number of targets to examine in neutrophils from critically ill patients. Neutrophils were taken from patients involved in the time-course study at time of study admission (see Methods section 2.03 d page 75), and their response to opsonised zymosan was examined. Figure V-26 demonstrates that phagocytosis was impaired to a similar degree to that seen in the VAP cohort (Figure III-1, page 100 above), However these cells were obtained within 48 hours of ICU admission in contrast to the median 8 days in the VAP cohort.

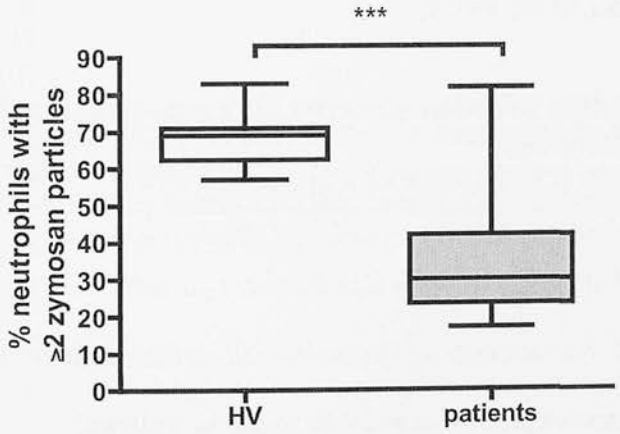


Figure V-26: Phagocytosis by patients' neutrophils is significantly reduced compared to healthy volunteers.
***P=0.001 by Mann-Whitney U-test. Data are shown as median (central line) inter-quartile range (box) and range (whiskers), (n=38 participants; 28 patients and 10 healthy volunteers).

Again, as seen in the VAP cohort (Figure IV-12, page 131), there was a significant correlation between neutrophil surface CD88 expression and phagocytosis (Figure V-27). As before no such correlation was found with other

markers of neutrophil activation, in that CD11b, CD16, CD64 and CD62L showed no significant correlation with phagocytosis (r=-0.05, 0.12,0.15, 0.01 respectively, all p values >0.05 by Spearman's Rho).

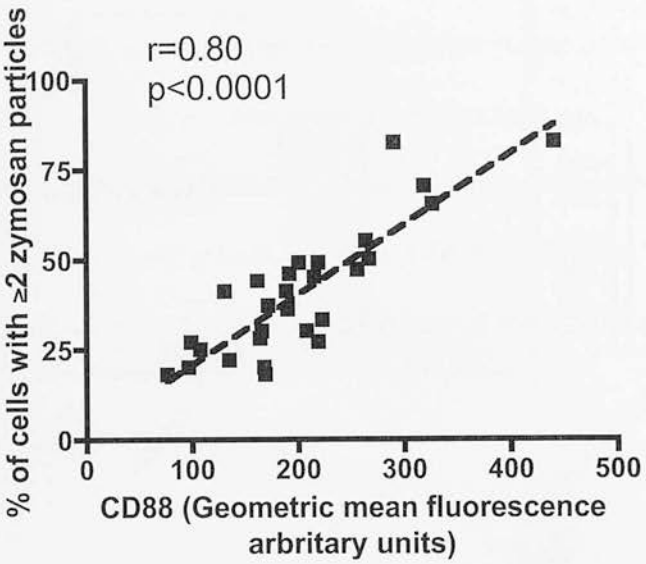


Figure V-27: Relationship between surface CD88 expression and phagocytosis by neutrophils.
n=28 individual patients. p and r values by Spearman's Rho.

Having confirmed the relationship between C5a exposure (as measured by CD88) and phagocytosis, the next step was to evaluate the mechanistic features determined during exposure of healthy neutrophils to rhC5a. Patients' neutrophils exposed to zymosan failed to activate RhoA (Figure V-28). Indeed, a small reduction was observed in RhoA relative to baseline, similar to that seen in rhC5a-treated cells (Figure V-14 page 158). Patients' neutrophils were also unable to effectively polymerise actin in response to zymosan (Figure V-29A, page 171), in keeping with the effects described for healthy neutrophils exposed to C5a (Figure V-15 page 159). Figure V-29B shows example data from the

flow cytometric analysis of actin polymerisation. In notable contrast to the results seen in healthy donor neutrophils (Figure V-4B, page 148) geometric mean fluorescence *falls* following exposure to zymosan.

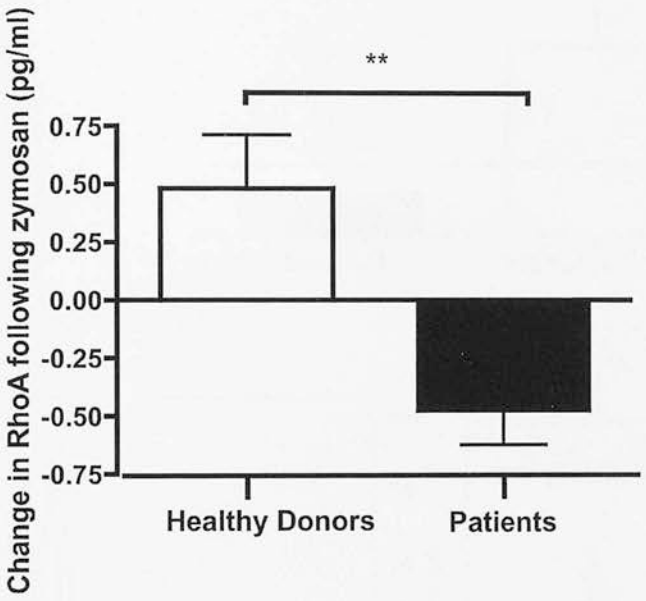
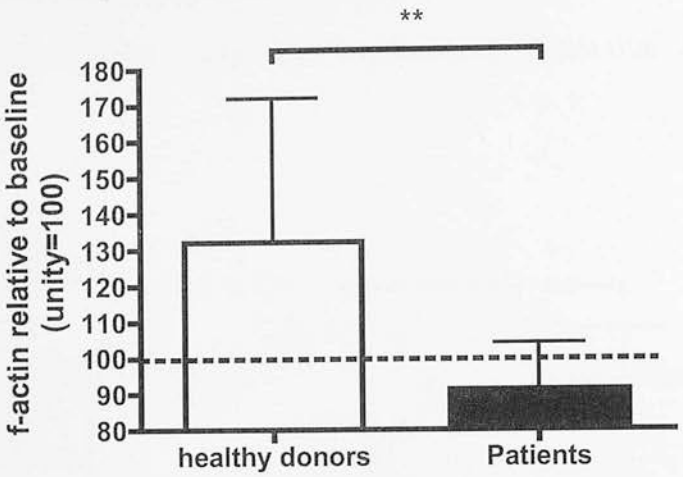


Figure V-28: RhoA activation in response to zymosan is impaired in patient neutrophils.
**P=0.002 by Mann-Whitney U-test. Data are shown as median and inter-quartile range (n=20; 10 patients and 10 healthy volunteers).

A



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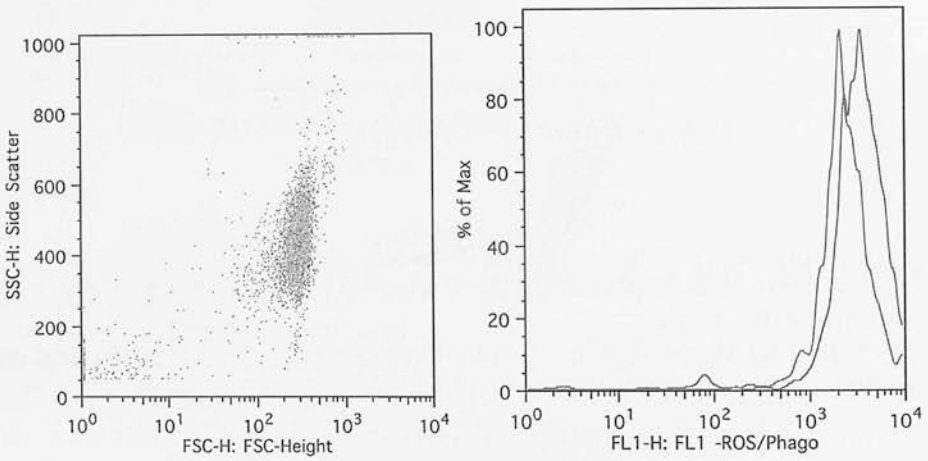


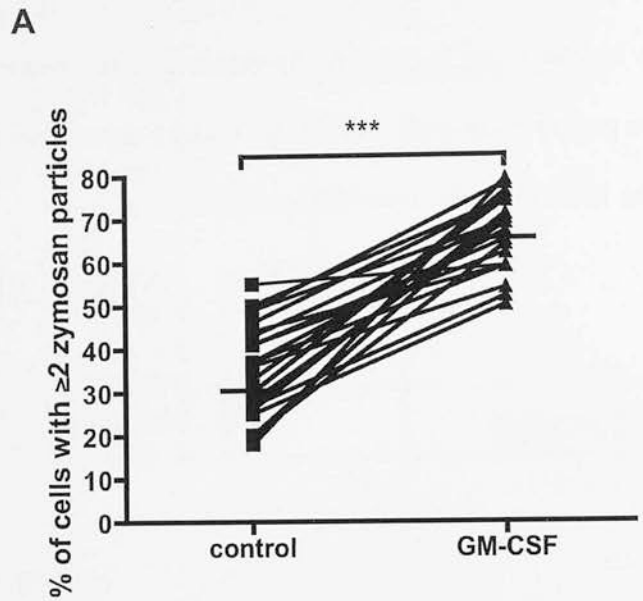
Figure V-29. Actin polymerisation by patient neutrophils in response to zymosan.

Panel A **Actin polymerisation following 5 minutes of zymosan exposure is impaired in patients**. **P=0.006 by t-test. Data are shown as mean and SEM (n=15; 10 patients and 5 healthy volunteers).

Panel B **Example data for actin polymersation by patient neutrophils.** The left-hand panel shows the forward/side scatter characteristics of the neutrophils, and the right-hand shows the FL-1 channel fluorescence (indicating degree of phalloidin staining) in unstimulated cells (red) and those exposed to zymosan for five minutes (blue). The geometric mean fluorescence for each is displayed in the box above.

(m) GM-CSF is able to 'rescue' phagocytosis by patient neutrophils.

In keeping with its ability to reverse rhC5a-induced phagocytic defects, *ex-vivo* application of GM-CSF to patient neutrophils was able to restore phagocytosis to levels similar to those seen in healthy volunteers (Figure V-30)



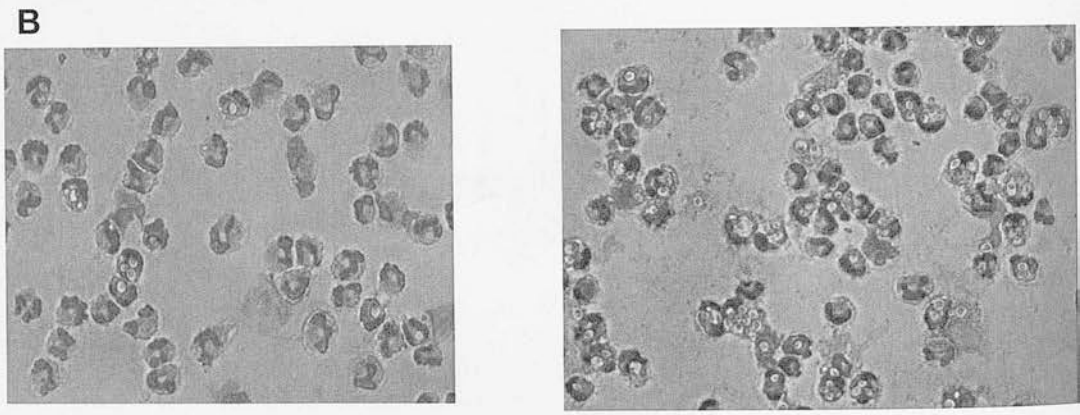


Figure V-30: GM-CSF restores phagocytosis in patients' neutrophils.
Panel A: **Data from patient neutrophils exposed to control or 0.3nM (5ng/ml) GM-CSF**. ***p<0.001 by Wilcoxon rank-sum test. Solid lines indicate median values (n=24 patients).
Panel B: **Example data from one patient's neutrophils**, illustrating the poor phagocytosis in the control treated cells and restored phagocytosis following GM-CSF. (X32 magnification)

Section 5.03 Chapter summary and discussion

Chapter 4 demonstrated the association between markers of C5a exposure and defects in neutrophil function. Chapter 5 expands on this by exploring the mechanism that underlies this defect. C5a binds to its major receptor, CD88, which activates the associated G-proteins. This in turn signals PI3Ks, both gamma and delta, to activate and act on their down-stream effector molecules. PI3K delta activation inhibits RhoA-GTP formation, consistent with work by other groups (Papakonstanti, Ridley & Vanhaesebroeck 2007), which then prevents the actin polymerisation response to phagocytic targets (i.e. zymosan). This results in the cell being unable to effectively phagocytose. Through mechanisms that remain partly opaque, GM-CSF is able to restore RhoA activation, actin polymerisation and phagocytosis. (Figures V-17 to 19). In a different group of patients from those explored in chapters 3 and 4, a similar degree of phagocytic defect was seen and the correlation between CD88 and phagocytosis confirmed (Figure V-27 page 169). Further strength was added to the supposition that the defect in patients is driven by C5a in the finding of close similarities in the intracellular response to zymosan, namely the impairment of Rho-GTP formation and actin polymerisation and the ability of GM-CSF to resurrect phagocytosis.

A potential weakness of this experimental approach relates to the relevance of the assay used to assess phagocytosis. Zymosan particles are derived from yeast (*Saccharomyces cerevisiae*) and are large compared to other phagocytic targets such as bacteria. Furthermore the use of cells adhered onto tissue culture plastic in IMDM may not fully reflect the physiological and pathophysiological

situations found in healthy and diseased humans. As such there remained a risk that the observations reflect *in vitro* artefacts specific to the model used. Therefore, a test of biological and clinical relevance was required. This forms the bulk of the next chapter, examining the relationship between C5a-mediated neutrophil dysfunction and a clinically significant outcome, namely acquisition of nosocomial infection.

Article VI. An enquiry into patterns of C5a-mediated neutrophil dysfunction in critically ill patients and its relevance to immunophenotyping and the acquisition of nosocomial infection

Section 6.01 Chapter overview and introduction

Chapter 5 demonstrated the mechanism by which C5a can impair the phagocytosis of zymosan, as well as indicating other potential impacts on neutrophil function. However the clinical relevance of these findings remained uncertain.

Although C5a-mediated neutrophil dysfunction is a novel finding in critically ill humans, it is not the only immune dysfunction characterised in this patient population. As shown in Chapter One, a variety of other immune cells are shown to be hypo-functional in sepsis and other forms of critical illness. It is therefore interesting to examine how C5a-mediated neutrophil dysfunction co-exists, if at all, with these other cellular defects.

Although as Chapter I (section 1.05 pages 51-58) makes clear, immune cells in various compartments display defects in functioning in critical illness, circulating cells are those which are destined for potential sites of infection and represent a key part of the body's response to any invading organism. Furthermore, cells in the blood which remain the most accessible both to researchers and clinicians, and hence findings from such cells are likely to be the most readily translatable into routine clinical practice. Therefore this chapter explores the patterns of

dysfunction in three cells, neutrophils, peripheral blood monocytes and T-cells (specifically the percentage of immuno-suppressive, regulatory T-cells). These are then related to the occurrence of nosocomial infection, the outcome measure chosen for immune suppression.

The hypothesis for this chapter is that C5a-mediated neutrophil dysfunction will exist alongside other immune dysfunctions, will predict risk of nosocomial infection and that patients with more immune dysfunctions will be at greater risk of infections.

This chapter takes each examined cell type in turn, starting with the neutrophil. Firstly the definition of dysfunction is established, followed by an exposition of the temporal patterns of such dysfunctions and then an examination of the relationship between the dysfunction and the acquisition of nosocomial infection. The final section is an examination of the effects of combining measures of immune dysfunction on the ability to predict infection acquisition.

I am grateful to Dr Niall Anderson for his help and advice on the statistical analysis of data presented in this chapter.

Section 6.02 Results

(a) Defining neutrophil dysfunction.

Assays of cellular function are informative, in that they indicate what the cells are actually doing in response to an immune challenge within a patient. However they are time consuming, require specialised equipment and procedures and may not be easy to standardise. Therefore proxy measures of function are

desirable, allowing more rapid throughput and assessment of a patient's current immunophenotype. To this end, using the data from Chapters 4 and 5, a readily assessable measure of C5a-mediated neutrophil dysfunction was derived.

As previously discussed, neutrophil surface CD88 expression varies in response to C5a exposure as well as showing a strong correlation with a key function i.e. phagocytosis (Figures IV-12, page 131 and V-27, page 169), and as such its use as a proxy measure of neutrophil dysfunction was evaluated. Section 5.02 a (page 143) demonstrated that after exposure to 10nM C5a, phagocytosis by healthy donor neutrophils drops below 50%. Section 4.02 h, (page 128) demonstrated that exposure to 10nM C5a induced an approximate 20% reduction in surface CD88 expression. 10nM C5a reduced CD88 expression from a mean of 319 arbitrary fluorescence units (AFU) to a mean of 249 AFU, hence a cut-off of 250 was chosen. A post-hoc examination of how this cut-off related to phagocytosis in patients was performed by regression analysis of the data presented in Figure V-26, (page 168) which revealed that a CD88 expression level of 250 AFU equated to a phagocytic capacity of 50% (Figure V-27, page 169) strikingly similar to the results achieved with in-vitro exposure to rhC5a (Figure V-1, page 144). A further examination of optimal cut-offs for CD88 is expounded below (Section 6.02 a).

(b) Recruitment of critically ill patients

Prior to recruitment starting a target was set by power calculation. An estimate of the rate of infection amongst patients staying for ≥48 was made using existing infection surveillance data that showed a rate of 25%. As there was a lack of

previous data on the effect of neutrophil dysfunction, an estimated doubling of the risk of nosocomial infection was assumed. Thus an intended recruitment of 92 would have 80% power at an alpha of 0.05 to detect such a difference, whilst 100 patients would have an anticipated event rate of 25, hence a recruitment target of 100 was selected. Ultimately a cohort of 96 patients was recruited. Figure VI-1 shows the consort diagram for recruitment. One patient had missing data on regulatory T-cells due to failure of flow staining, so was excluded leaving 95 patients to enter the final analysis.

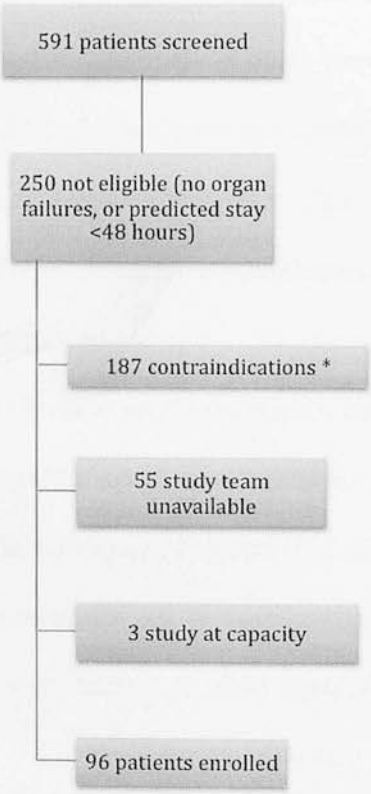


Figure VI-1: Consort diagram showing screening and recruitment of patients.
* Reasons for exclusion are as follows; 50 consent not available or declined, 49 in an incompatible study, 43 receiving immunosuppressants other than cortico-steroids, 20 not expected to survive >24 hours, 12 confirmed or suspected H1N1, 5 previously in study, 4 being transferred to another unit in <48 hours, 2 HIV, 2 pregnant.

(c) Patterns of CD88 expression over time

At study admission (i.e. within 48 hours of ICU admission) the median CD88 was 230 AFU (IQR 167-286), with 57 patients' neutrophils (60%) being below the cut-off of 250. The scatter-gram of CD88 values at the point of entry to the study is shown in Figure VI-2. By study day 4, 63 patients remained in ICU without having acquired infection, of whom 54 (86%) were below the cut-off of 250. Figure VI-3 shows the distribution of CD88 values at each time point for all patients, restricting this to the 23 patients with samples until day 8-10 showed a similar downward trend in median CD88 levels, although individual patients demonstrated various patterns of rising, falling or static CD88 values (data not shown).

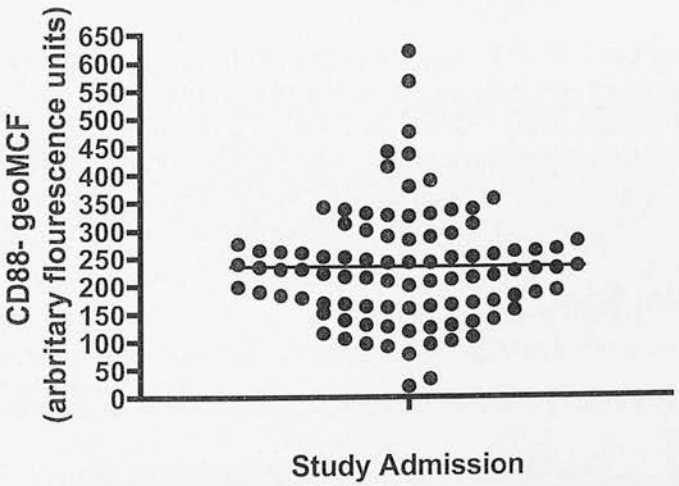


Figure VI-2: Scatter-gram of neutrophil surface CD88 values on admission to the study.
n=95 patients, solid line indicates the cut-point for dysfunction (250 arbitrary fluorescence units)

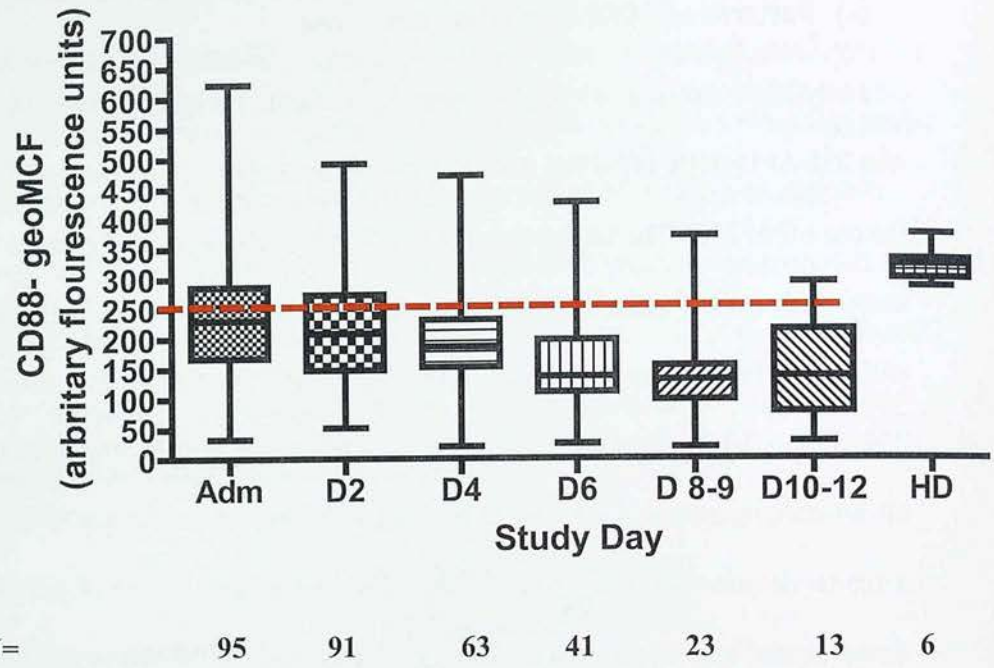


Figure VI-3: Neutrophil surface CD88 expression at various time points.
Data shown as median (central line), inter-quartile range (box) and range (whiskers), line under graph indicates number of samples from each time point n=6 healthy donors (HD). CD88 expressed a geometric mean fluorescence (geoMCF) in arbitrary units. ADM=study admission (within 48 hours of ICU admission). $P<0.001$ by Kruskal-Wallis ANOVA, all study time points differ significantly from healthy donors (HD, $P<0.05$ by Dunn's post-hoc test). Hatched red line indicates the cut off for 'dysfunction' derived below.

To permit analysis a summary measure was developed, allowing patients to be dichotomised into those with 'dysfunction' and 'no dysfunction'. Patients who were admitted with CD88 ≤ 250 AFU and remained below 250 AFU throughout were classified as 'dysfunction', similarly those who were admitted with CD88 >250 AFU but fell below 250 AFU, censored for 2 days prior to event in those acquiring infection, were classified as 'dysfunction'. Those admitted with CD88 >250 AFU and who remained >250 AFU throughout were classified as 'no dysfunction', similarly those admitted with CD88 ≤ 250 AFU but rising above

250 were classified as 'no dysfunction'. Figure VI-4 shows this dichotomisation in diagrammatic form.

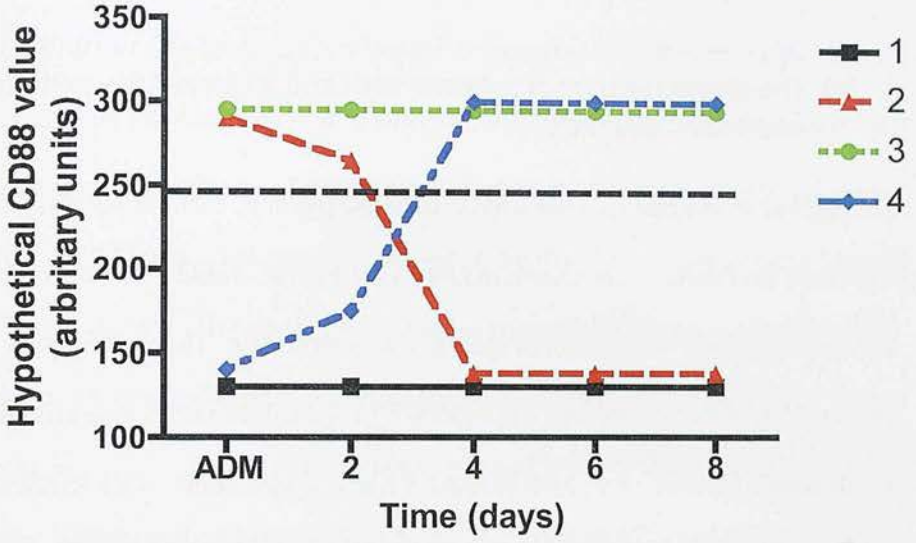


Figure VI-4: Illustration dichotomising patients into 'dysfunction' or 'no dysfunction' groups.
Hatched line at 250=cut off for dysfunction. Example 1, all of a given patient's results below cut-off=dysfunction. Example 2, values from a patient start high but fall below the cut-off =dysfunction. Example 3, all of a given patient's results are above the cut-off=no dysfunction. Example 4, a patient's values start low but rise to above the cut off before any infection occurring=no dysfunction. ADM=study admission (within 48 hours of ICU admission)

Overall, 69 patients fell into the 'dysfunction' group, whilst 26 were in the 'no dysfunction' group. 83% of patients remained in the same group as admission, with 16 (17%) changing group; 13 moving from CD88>250 to ≤ 250, whilst 3 moved from ≤ 250 to >250. Eleven changes occurred within the first two days

of the study, and 13 within the first 4 days, with only 3 changes occurring later on in the admission.

(d) The characteristics of patients with and without C5a-mediated neutrophil dysfunction

Demographic and clinical characteristics of the patients, dichotomised by CD88-indicated dysfunction, are shown in table VI-1 below. Table VI-2 shows the outcomes, namely acquisition of infection and mortality. Those with C5a-mediated dysfunction showed a 5.4 (95% CI 1.4-21.0) times relative risk of nosocomial infection compared to those without dysfunction. As a sensitivity analysis, the effect was compared excluding those with only 'probable' infections. This revealed a relative risk increase of 4.6 (95% CI 1.2-18.0, p=0.008 by Fisher's exact test), which did not differ significantly from the combined 'confirmed' and 'probable' infections analysis. Similarly, excluding those patients who shifted from one CD88 classification to another had no significant effect on relative risk of nosocomial infection (RR 4.7, 95% CI 1.2-18.3, P=0.007 by Fisher's exact test).

To examine the accuracy of the pre-defined cut-off point for CD88 (i.e. 250AFU) a receiver operator characteristic (ROC) curve was constructed, comparing the sample most temporally related to infection (censored for two days prior) with samples from patients who did not develop infection. Youden's method (Youden, 1950) indicated an optimal cut-off point of 246AFU, justifying the pre-defined definition.

Table VI-1 contains details concerning the various risk factors set out in Chapter 1 (table I-2, page 23) that have been previously identified as being associated with nosocomial infection (reviewed by Vincent JL, 2003). Significant differences in these factors between the two groups could, therefore, confound any effects of C5a-mediated neutrophil dysfunction on nosocomial infection. Of note data on smoking (current or stopped within previous year) and alcohol use (defined as >10 units or <10 units consumption /week) are not included in the table as these data were poorly recorded in the ICU notes (data present for only 58% of patients). However amongst those patients for whom it was recorded there were no significant differences between the two groups (alcohol >10units occurring in 47% and 42% of patients with and without dysfunction, p=1 by Fisher's exact test.; a history of smoking occurring in 47% and 48%, p=1 by Fisher's). Outcomes in terms of acquisition of nosocomial infection and mortality are shown in table VI-2 below.

Variable	Dysfunction	No Dysfunction	P value
N=	69	26	
median (range) age	57 (43-71)	62 (46-72)	0.71 *
% male	62%	66%	0.8 §
% admitted with sepsis	44%	44%	1 §
% surgical patients	25%	26%	1 §
% trauma patients	1%	12%	0.07 §
% receiving corticosteroids	28%	15%	0.29 §
% transfused	54%	27%	0.04 §
comorbid conditions (median/IQR Charlson index)	3 (1-4.5)	3 (1-4)	0.74 *
% with diabetes mellitus	20%	15%	0.78§
% with chronic lung disease	17%	11%	0.75§
% intubated	88%	92%	0.7 §
% central venous catheter	88%	92%	0.7 §
% urinary catheter	94%	92%	0.66 §
% tracheostomy	17%	4%	0.11 §
% receiving parenteral nutrition	22%	20%	1§
% receiving renal replacement therapy	23%	46%	0.06 §
median (IQR) APACHE II Score	21 (19-28)	23 (15-29)	0.95 *
median (IQR) admission SOFA score	9 (7-11)	6 (4-9)	0.003 *
median (IQR) peak SOFA score	9 (8-13)	6 (4-10)	0.002*

Table VI-1: Demographic and clinical details for patients with and without C5a-mediated neutrophil dysfunction.
IQR-inter-quartile range, SOFA sequential organ failure assessment, APACHE II –acute physiology and chronic health evaluation II, * p value by Mann-Whitney, § p value by Fisher's exact test.

Outcome	Dysfunction	No dysfunction	P value (Fisher's exact test)
% acquiring infection in ICU	43%	8%	0.001
% crude unit mortality	29%	15%	0.2
% crude hospital mortality	36%	27%	0.12
Standardised mortality ratio (SMR)	0.72	0.62	1.0

Table VI-2: Outcomes for patients with and without dysfunction.
SMR calculated from APACHE II predicted mortality

Amongst patients in the dysfunction group there was significantly increased C3a concentrations over time, indicating persistent complement activation. In contrast, patients in the no dysfunction group showed a trend towards reducing C3a levels (Figure VI-5). This dichotomised pattern was not replicated for any of the cytokines examined (with the levels remaining unchanged or decreasing in both groups for IL-1β, 6, 8, 10 and TNF-α) and for the marker of neutrophil activation sCD62L (table VI-3). C5a rose by a small amount in both groups, although the rise was not significant in either (median rise 9ng/ml in the dysfunction group ($p=0.058$) and 2ng/ml in no dysfunction group ($p=0.1$, both p values by Wilcoxon rank sum test). As an alternative analysis, peak values for each of the cytokines, anaphylatoxins and soluble receptors were compared between patients with and without dysfunction (table VI-4). C3a was the only molecule that showed significantly higher levels in the dysfunction group than the non-dysfunction group, mirroring the results of the change over time analysis

above. There were non-significant trends to higher levels of IL-8 and sCD62L in the dysfunction group, but both IL-1β and TNF showed higher levels in the non-dysfunction group suggesting that pro-inflammatory activation was not restricted to one group of patients or the other.

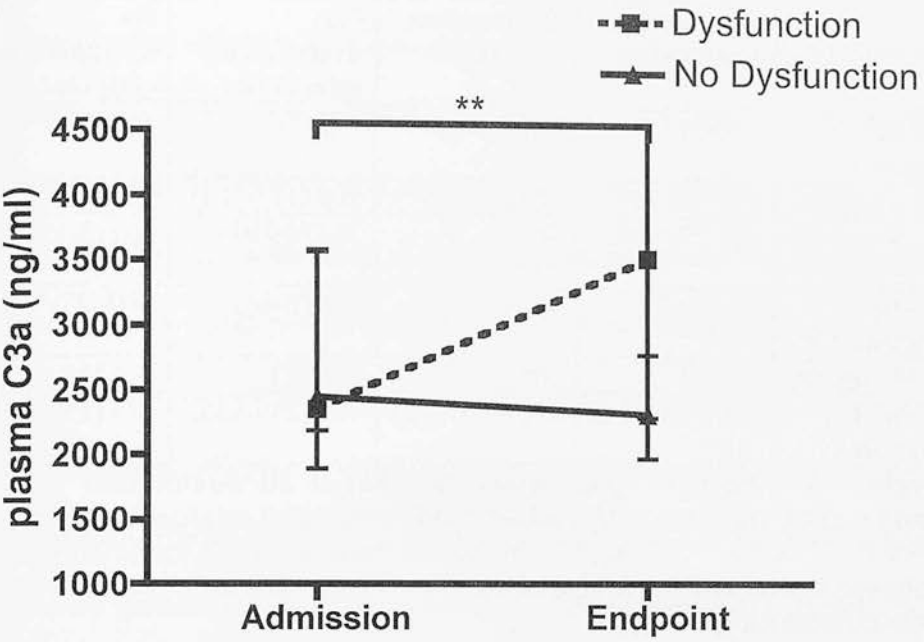


Figure VI-5: Change in C3a levels between admission and study endpoint.
Endpoints being infection, death or discharge –in the case of infection censored for 2 days prior to infection) in patients with and without CD88 defined neutrophil dysfunction. N=91 patients, 69 with dysfunction and 22 without (4 patients had only one sample so could not be included in this analysis). ** P=0.01 by wilcoxon rank sum test.

	Dysfunction –admission	Dysfunction –endpoint	No dysfunction-admission	No dysfunction-endpoint
IL-1β (pg/ml)	2 (0-5)	1(0-5)	4 (2-7)	5(3-8)
IL-6 (pg/ml)	242 (82-639)	90 (42-285)**	200 (113-521)	92 (39-168)**
IL-8 (pg/ml)	122 (59-344)	66 (34-202)***	78 (36-202)	40 (18-121)**
IL-10 (pg/ml)	25 (14-75)	17 (9-39)***	25 (16-46)	14(8-27)**
TNF (pg/ml)	2 (0-7)	3(0-8)	6(2-12)	5(3-11)
sCD62L (ng/ml)	456 (331-714)	494 (329-712)	402 (259-636)	415 (259-574)

Table VI-3: Change in cytokine levels between admission and endpoint for patients with and without neutrophil dysfunction.
*P<0.05, **<0.01, ***<0.0001 by Wilcoxon Rank Sum test for difference between admission and endpoint sample. Values shown as median and inter-quartile range.

Cytokine	Dysfunction	No dysfunction	Healthy volunteer comparator group	P value
C3a (ng/ml)	4206 (2637-5500)	2686(2319-4325)	2570 (1285-2785)	0.03
IL-1β (pg/ml)	3(0-8)	7 (3-9)	0 (0-6)	0.03
IL-6 (pg/ml)	364 (119-1021)	200 (116-521)	0 (0-11)	0.22
IL-8 (pg/ml)	165 (66-464)	91 (36-209)	0 (0-7)	0.06
IL-10 (pg/ml)	31 (18-100)	31 (16-64)	0 (0-4)	0.95
TNF (pg/ml)	4 (0-10)	7 (4-13)	0 (0-2)	0.02
sCD62L (ng/ml)	679 (460-1031)	545(375-703)	NA	0.06

Table VI-4: Peak cytokine levels in patients with and without neutrophil dysfunction.
P value is by Mann-Whitney U-test between patient groups. Values shown as median and inter-quartile range. Healthy volunteer values are provided for comparison purposes.

(e) C5a-mediated neutrophil dysfunction as a predictor of nosocomial infection

Although C5a-mediated neutrophil dysfunction is clearly associated with a significant increase in the risk of acquiring nosocomial infection in the ICU, it

remains a possibility that this effect may result from the influence of confounding variables. Two possible groups of confounders present themselves, firstly 'duration of exposure', i.e. length of time in ICU, and secondly the risk factors identified in table VI-1 above as significantly different between the two groups. To analyse the effect of these a Cox hazards model was constructed, including all variables with a significant difference between the 'dysfunction' and 'no dysfunction' groups although using a more liberal definition of 'significance' of $p \leq 0.1$ to avoid excluding factors with a clear trend but which narrowly avoided the standard significance cut off of <0.05. Accordingly the factors included alongside 'dysfunction', were 'admission with trauma', 'blood transfusion', 'renal replacement therapy' and 'peak SOFA score'. Admission SOFA and peak SOFA showed significant co-linearity ($r^2=0.8$), hence only one measure was chosen. Table VI-5 shows the results of the analysis. Only 'dysfunction' showed any significant relationship with the acquisition of infection. Exclusion of co-variates with p value of >0.1 by backwards stepwise removal results in a model containing dysfunction alone, with an overall p value of 0.036.

Figure VI-6 shows the Kaplan-Meier plot for infection in patients with and without dysfunction.

	P Value	Hazard ratio (95% Confidence Interval)
'Dysfunction'	0.05	4.1 (1.0-17)
Blood Transfusion*	0.54	1.5 (0.7-3.4)
Trauma	0.91	1.3 (0.3-3.9)
Renal replacement therapy	0.18	0.63 (0.3-1.4)
Peak SOFA score	0.22	1 (0.9-1.1)

Table VI-5: Hazard ratios and P values for the variables included in the Cox hazards model of infection acquisition.
* Blood transfusion was included as a time-dependent co-variate as transfusion occurred at differing times during admission, and so violated the 'proportional' assumption.

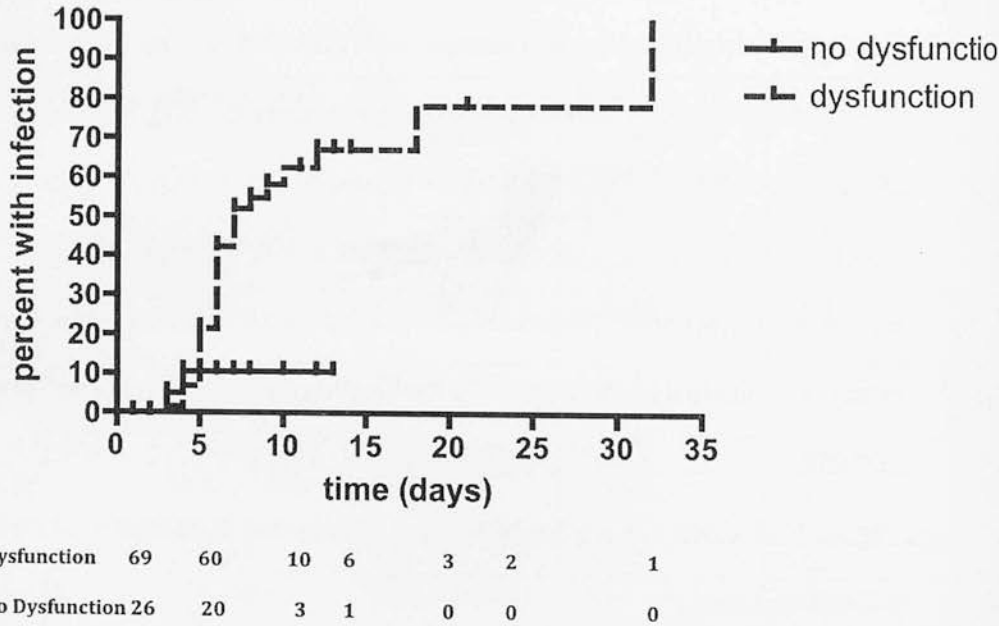


Figure VI-6: Kaplan-Meier plot for patients with and without neutrophil dysfunction.
P=0.04 by log-rank test. Numbers underneath indicate numbers remaining in alive in ICU without infection at each time point.

(f) Relationship between CD88 and infection.

In the VAP cohort (Chapters 3 and 4) the blood sample was taken on the day infection was diagnosed, so preventing assessment of whether the infection preceded the dysfunction or vice-versa. In the prospective cohort described in this Chapter samples were censored for 2 days prior to infection to try and minimise the effect of any infection on the results. To further test the potential relationship between infection and CD88, surface expression was compared in patients admitted with and without sepsis (figure VI-7), and showed no significant difference.

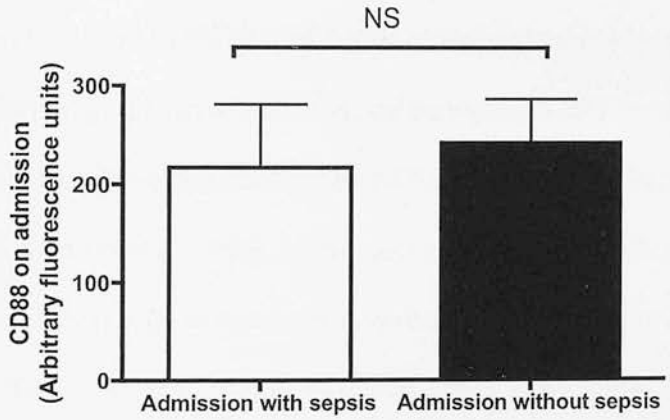


Figure VI-7: CD88 expression amongst patients admitted with and without sepsis. Data shown as median and inter-quartile range. n= 95 patients, 42 with and 53 without sepsis. NS, p=0.5 by Mann-Whitney.

The prospective cohort also allowed assessment of CD88 levels before and after infection, comparing all those who developed infections and showing the trends over time in this group (Figure VI-8).

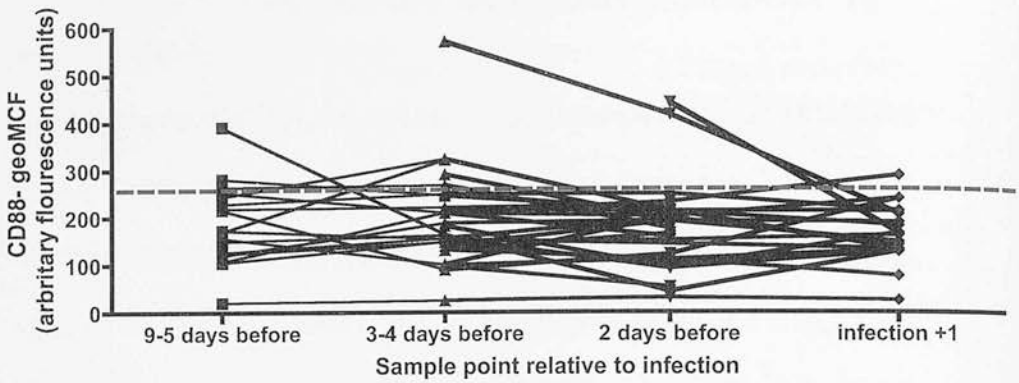


Figure VI-8: CD88 expression before and after nosocomial infection. Data from n=33 patients acquiring infections, p=0.74 by Kruskal-Wallis ANOVA. Hatched line indicates the cut-off value for 'dysfunction'.

Interestingly, analysis of those patients admitted to ICU with sepsis sub-divided into those with hospital-acquired and those with community-acquired infections showed a divergence in CD88 expression (figure VI-9). All patients admitted to ICU with hospital-acquired infection had CD88 levels below the 250 AFU cut-off.

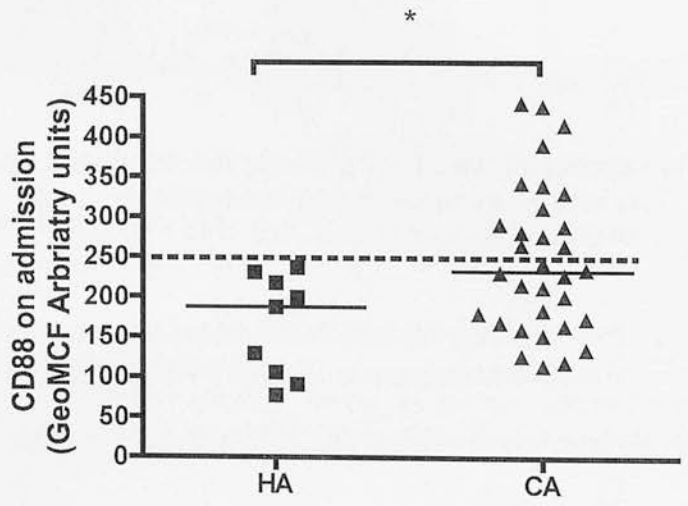


Figure VI-9: CD88 expression in patients admitted with hospital-acquired (HA) or community-acquired (CA) sepsis.
Solid lines indicate median values. Hatched line indicates 250 AFU cut-off for neutrophil dysfunction. N=42 patients, 9 with HA and 33 with CA sepsis. *p=0.02 by Mann-Whitney U test.

(g) Other immune dysfunctions

Several other problems with the immune system have been examined in the context of sepsis and critical illness (see section 1.05, pages 51-60). Two of these were examined in the prospective study cohort. First was monocyte deactivation, by measuring surface HLA-DR expression (Höflich et al. 2002), and the second was the percentage of regulatory T-cells (Venet et al. 2009). These are discussed in more detail in the following sections.

(h) Monocyte deactivation

Several groups have now reported the presence of 'deactivated' monocytes in the peripheral circulation of patients with sepsis (Döcke et al. 1997), trauma (Asadullah et al. 1995) and fulminant hepatic failure (Berry et al. 2011). The cardinal feature of monocyte deactivation is the inability of the cells to mount an effective TNF-α response to a challenge of LPS. This was also demonstrated in the sub-set of patients in the VAP study in whom monocyte function was examined (see section 3.02 e, page 102). Baehr and colleagues demonstrated that monocyte HLA-DR expression could act as a marker for monocyte deactivation (Baehr et al. 1989).

In the 95 patients in the prospective cohort, monocyte HLA-DR expression was determined as per the Methods section 2.03 l (page 87), using QuantiBRITE beads (BD Bioscience) to provide quantification of PE molecules per cell. Meisel and colleagues used the same technique to guide GM-CSF therapy in critically ill adults (Meisel et al. 2009), and suggested a cut-off of 8000

molecules/cell for monocyte deactivation. Figure VI-10 shows the patterns of HLA-DR expression over time. Patients showed depressed median HLA-DR expression relative to healthy donors at all time points sampled.

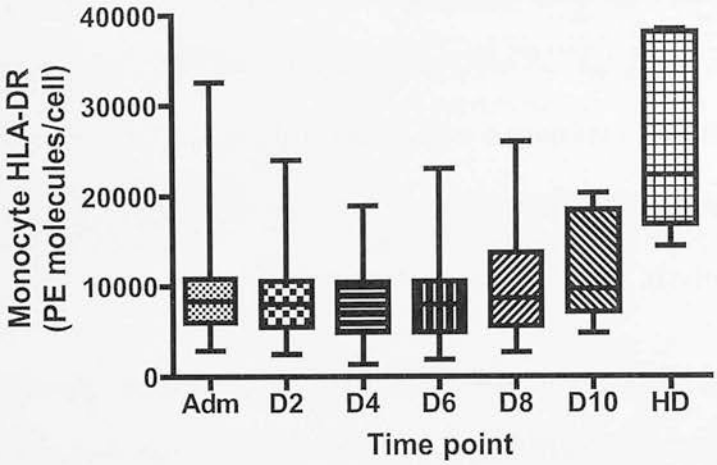


Figure VI-10: HLA-DR expression by peripheral blood monocytes. Data shown as median (central line), inter-quartile range (box) and range (whiskers), from n=95 patients at multiple time points and n=6 healthy donors (HD), numbers at each time point as per Figure VI-3 page 181. P=0.0013 by Kruskal-Wallis ANOVA, Dunn's post hoc test <0.01 for patients vs. healthy donors at all time points except D8 (P<0.05) and D10 (P>0.05).

(i) Deriving a cut-off and categorising monocyte 'deactivation'

As with neutrophil CD88, patients were divided into those with and without monocyte deactivation on the basis of whether their results fell above or below the cut-off. Those who changed from one side of the cut-off to the other were categorised in the same way as for CD88 (Figure VI-4, page 182).

Initial analysis of the patients using the cut-off proposed by Meisel (Meisel et al. 2009) of 8000 revealed 42 patients with monocyte deactivation. Using this cut-off to define deactivation produced a relative risk of infection of 1.34 (95% CI 0.7-2.3, P=0.39) compared to those without dysfunction.

To determine whether this was the optimal cut-off, a ROC curve was constructed, comparing the sample most temporally related to infection (censored for two days prior) with samples from patients who did not develop infection. Youden's method indicated an optimal cut-off point of 10,000 molecules per cell.

This adjusted cut-off resulted in 62 patients being classified as having 'deactivated' monocytes and 33 having 'no deactivation'. 22 patients changed groups during their stay (11 moving from 'deactivation' to 'no deactivation' and 11 moving in the other direction). Using this adjusted cut-off the relative risk of infection was 3 (95%CI 1.3-6.9, p=0.0035 by Fisher's exact test).

(j) Regulatory (CD4+,CD25+,FoxP3+) T-cells

As discussed in the Introduction (section 1.05 e page 55), work by Monneret and colleagues (Venet et al. 2009) has demonstrated the presence of elevated levels of immuno-suppressive regulatory T-cells (T-regs) in patients with sepsis. They have also demonstrated an association between elevated levels of these cells and mortality in patients with sepsis and septic shock (Huang et al. 2010, Monneret et al. 2003).

Levels of T-regs, expressed as a percentage of total CD4+ve T-helper (T_H) cells, were determined in patients. As shown in figure VI-11 there were higher levels of T-regs in patients than in healthy individuals, although the difference only became significant at day 6. As the previous work had shown T-regs to be elevated in sepsis, patients were dichotomised into those with and without sepsis

on admission for analysis of T-reg levels. Figure VI-12 shows the data from figure VI-11 dichotomised for the presence or absence of sepsis on admission.

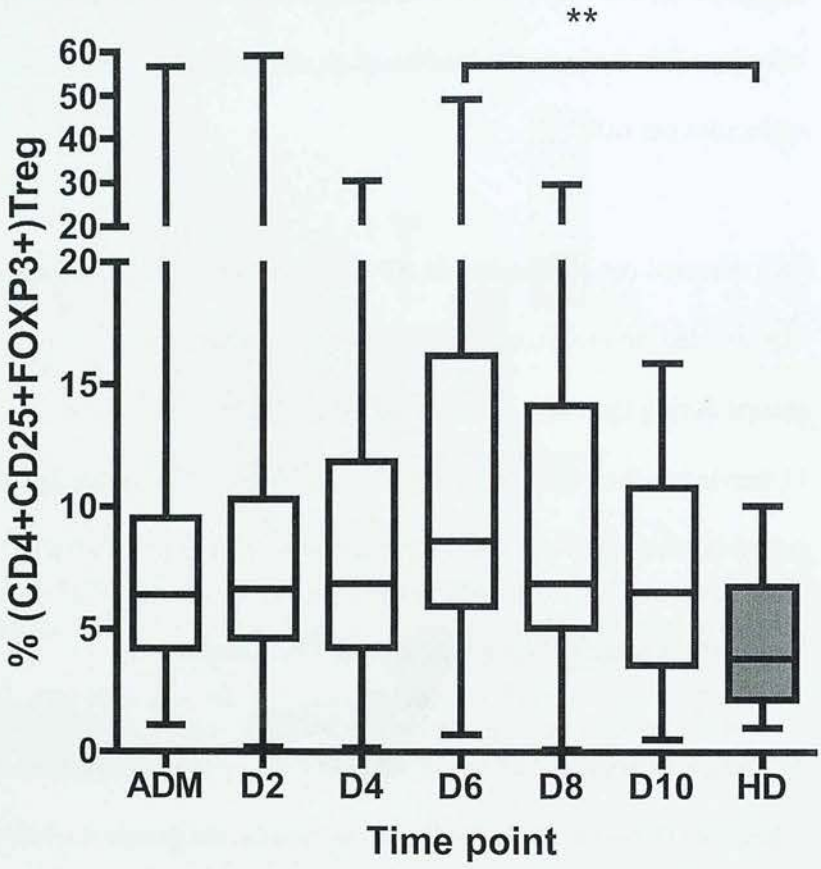


Figure VI-11: T-regs as a percentage of all CD4+ T_H cells.
Data shown as median (central line), inter-quartile range (box) and range (whiskers), from n=95 patients at multiple time points and n=6 healthy donors (HD), numbers at each time point as per Figure VI-3 page 181. P=0.027 by Kruskal-Wallis ANOVA, ** p<0.01 by Dunn's post hoc test for patients vs. healthy donors.

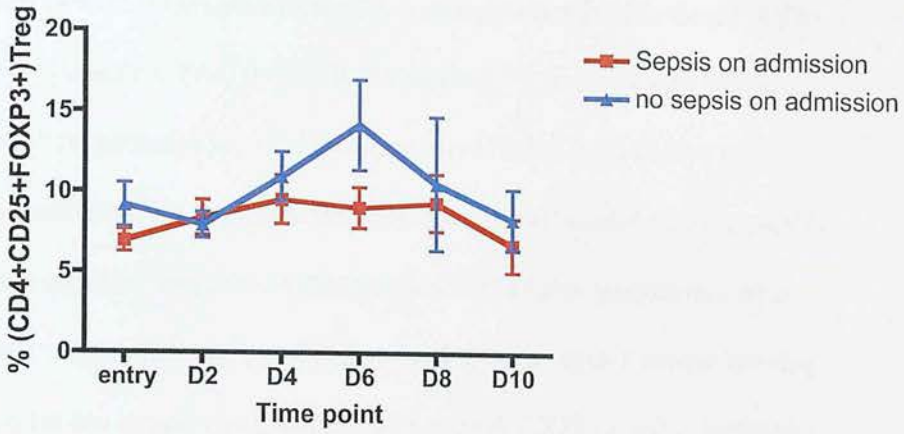


Figure VI-12: T-reg levels by admission diagnosis.
Data shown as mean and SEM. N=95 patients, 42 with sepsis and 53 without. P=0.11 for difference between the groups by two-way ANOVA.

It is therefore apparent that T-reg levels do not differ significantly between patients with and without sepsis, and therefore likely that the elevated levels represents a reaction to the inflammatory state that occurs in both septic and non-septic critical illness.

(k) Setting a cut-off for 'elevated' T-reg cells

Amongst the healthy volunteers the median percentage of T-reg cells was 3.8%, with a maximum of 10%. A similar maximum of 10% was found by Monneret (Venet et al. 2009), with similar values found in other work (Taams et al. 2001, Stephens et al. 2001) Therefore 10% was set as the cut-off for 'normal' levels of T-reg cells.

Thirty-seven patients had T-regs which rose above 10% prior to discharge, forming the 'T-reg high' group whilst 58 were in the 'T-reg normal' group. In contrast to HLA-DR and CD88, patients whose levels rose above 10% were often in the 'normal' group at admission, 26 patients moving from 'normal' to

'high' levels of T-regs during admission, whilst 8 patients moved from 'high' to normal.

Those patients whose T-regs rose above 10% prior to discharge had a relative risk for developing infection of 2.4 (95%CI 1.3-4.2, p=0.002) compared to those patients whose T-regs remained below this level. As before the cut-off point was examined using an ROC curve, which produced an optimal cut-off point of 9.8%, close to that derived from using the healthy volunteer maximum.

(I) Details of ICU-acquired infections

Thirty three (35%) patients developed confirmed or suspected infection, 26 confirmed and 7 probable. The site of these infections is detailed in table VI-6.

Confirmed infections	
	Pneumonia- 10 (7 ventilator-associated)
	Blood stream infections - 4
	Catheter-related blood stream infections -3
	Urinary tract infections- 5
	Surgical site/soft tissue infections-4
Probable infections	
	Pneumonia- 4 (all VAP)
	Intra-abdominal infection- 3

Table VI-6: Site of infections acquired in ICU.

In total 20 patients underwent adjudication panel review, 5 were ruled 'confirmed', 7 'probable' and 8 'unlikely'. The organisms cultured are shown in table VI-7. Of the non-confirmed adjudications, 6 of the 7 'probable' infections had either negative cultures with other strong clinical evidence of infection or did not have cultures taken as care was being withdrawn but with strong clinical

evidence. The remaining 'probable' patient grew bacteria below the 10^4 CFU/ml cut off for quantitative BAL fluid. Of the 'unlikely' infections, two had negative cultures with no potential focus of infection identified, 5 had positive cultures without any evidence of infection (termed 'colonisation') and 1 had persistence of the same organism cultured on admission with a change in antibiotic sensitivities.

Infection category	Organism	Frequency
Confirmed	*Burkholderia cepacia*	1
	Citrobacter braakii	1
	Enterobacter cloacae	3
	Escherichia coli	5
	Coliform –no further specification	1
	Enterococcus faecalis	2
	Klebsiella pneumoniae	2
	Haemophilus influenzae	1
	Pseudomonas aeruginosa	3
	Staphylococcus aureus	3
	Coagulase negative *Staphylococci*	1
	Streptococcus pneumoniae	1
	Other *Streptococci*	1
	Anaerobes	1
	Candida albicans	4
	Herpes simplex	1
Probable	Culture negative	4
	No samples taken as care withdrawn	2
	Staphylococcus aureus	1
Unlikely	Culture negative	2
	Acinetobacter baumannii	1
	Haemophilus influenzae	1
	Klebsiella pneumoniae	1
	Coagulase negative *Staphylococci*	2
	Streptococcus pneumoniae	1
	Staphylococcus aureus	2
	Candida albicans	1

Table VI-7: Culture results from patients with confirmed, suspected and unlikely infections.
Some patients grew more than one organism from the site of infection.

Of the patients acquiring infection in the ICU, 12 had sepsis on admission. None of these grew the same organism on admission and following the acquisition of infection. Only one patient developed infection at the same site as the site of admission infection, a patient admitted with *Varicella zoster* pneumonia who developed *Klebsiella pneumoniae* ventilator-associated pneumonia 6 days after admission. It therefore seems unlikely that infections present at admission were mistaken for nosocomial infection.

(m) Characteristics of patients developing infection.

The sections above have demonstrated that a majority of critically ill patients have C5a-mediated neutrophil dysfunction and that this is associated with an increased risk of nosocomial infection. This risk appears to be independent of other established risk factors. There remains the possibility that patients who develop infections are different in other ways from those who do not, and therefore an analysis was undertaken of the clinical and demographic features of those patients who did and did not develop infection.

Variable	Infection	No infection	P value
N=	33	62	
median (range) age	60 (20-88)	59 (16-85)	0.92*
% male	61%	63%	0.41§
% admitted with sepsis	34%	50%	0.20§
% surgical patients	30%	23%	0.46§
% trauma patients	3%	5%	0.65§
% receiving corticosteroids	30%	23%	0.46§
% transfused	52%	44%	0.52§
Comorbid conditions (median/IQR Charlson index)	3 (1.5-4)	3 (1-5)	0.42*
% with diabetes mellitus	21%	11%	0.78§
% with chronic lung disease	6%	21%	0.08§
% intubated	97%	85%	0.16§
% central venous catheter	97%	85%	0.16§
% urinary catheter	100%	90%	0.09§
% tracheostomy	9%	6%	0.70§
% receiving parenteral nutrition	30%	15%	0.1§
% receiving renal replacement therapy	42%	39%	0.83§
median (IQR) APACHE II Score	21 (18-26)	23.5(18.5-29)	0.30*
median (IQR) admission SOFA score	9 (6.5-11)	8 (4-10)	0.20*
median (IQR) peak SOFA score	9 (8-12.5)	8 (5-11)	0.16*
% with neutrophil dysfunction	94%	62%	0.0007§
% with monocyte deactivation	85%	55%	0.003§
% with elevated T-regs	61%	27%	0.0016§

Table VI-8: Demographic and clinical features of patients who developed nosocomial infection and those who did not.
*P by Mann-Whitney, § P by Fisher's exact test

(n) Immune dysfunction and nosocomial infection

In sections 6.02 a-f the prevalence and impact of C5a-mediated neutrophil dysfunction is discussed, noting its impact on acquisition of nosocomial

infection. Sections 6.02 g-k show that this association is not confined to neutrophils, but also extends to deactivated monocytes and elevated levels of T-regs. With these three factors being the only ones which differed significantly between patients with and without nosocomial infection in the simple analysis in table VI-8.

To examine whether the effects of each of these three immune defects were additive or simply occurred together, several analyses were conducted. First was an analysis of co-linearity between the three dysfunctions. None of the three had a correlation co-efficient of >0.25, indicating sufficient independence for entry into a multiple regression model. Second, the three were placed in a binary logistic regression model with infection as the dependent variable. The results are shown in table VI-9 below, with all three retaining their independent predictive effect.

Parameter	Odds ratio (95%CI) of acquisition of infection	P value
Neutrophil dysfunction	7.6 (1.5-37)	0.013
Monocyte deactivation	4.7 (1.4-15.7)	0.013
Elevated T-regs	4.3 (1.6-12.1)	0.004

Table VI-9: Logistic regression model for the relationship between individual immune dysfunctions and the acquisition of nosocomial infection
Interaction significant at P<0.01 by binary logistic regression

Finally the effect of cumulative occurrence of immune dysfunction was analysed. Although the point estimates for the relative risk (and regression-derived odds ratios) for nosocomial infection differed between the dysfunctions, their confidence intervals overlapped to a high degree, precluding any assignment of

differential risk. As a result a value of '1' was applied to each dysfunction, and the patients analysed by whether they had 0,1,2 or 3 dysfunctions (table VI-10).

Number of dysfunctions [a]	N=	% acquiring nosocomial infection (95% CI)
0	11	0 (0-0%)
1	21	10% (0-22%)
2	43	37% (23-52%)
3	20	75% (56-94%)

Table VI-10: Relationship between burden of immunodysfunction and acquisition of nosocomial infection.
(i.e. C5a-mediated neutrophil dysfunction, monocyte deactivation and elevated regulatory T-cells). P=0.0004 by Chi squared test for trend.

(o) Interaction between immune dysfunction and other risk factors for infection.

As noted in Section 1.02 c (page 18) multiple factors have been associated with nosocomial infection. It remains possible that the apparently strong and additive effects of immune dysfunctions are simply epiphenomena of other established risk factors for infection. To examine this a Cox hazards model was constructed. To select the variables to be included in the model univariate analysis was performed for relationship between the variable and acquisition of infection (Table VI-11), with variables with a p-value of <0.1 being entered into the model. Given the variability of T-reg counts and their propensity to change from 'high' to 'low' or vice versa during stay, the model was constructed with T-regs as a time-dependent variable.

The results of the model are shown in table VI-12 below, demonstrating that both C5a-mediated neutrophil dysfunction and elevated T-reg cells remained significant predictors of infection whilst monocyte deactivation lost its

significance (p=0.07). Both the clinical predictors of infection lost their significant effect once they entered the model.

	Variable	Infection	No infection	P value for univariate analysis
Pre-morbid factors	comorbid conditions (median/IQR Charlson index)	3 (1.5-4)	3 (1-5)	0.44
	% with chronic lung disease	6%	21%	0.2
	% with diabetes mellitus	21%	11%	0.89
	median (range) age	60 (20-88)	59 (16-85)	0.77
	% male	61%	63%	0.82
Admission factors	median (IQR) admission SOFA score	9 (6.5-11)	8 (4-10)	0.27
	median (IQR) admission APACHE II Score	21 (18-26)	23.5(18.5-29)	0.55
	admission with shock	**77%**	**56%**	**0.07**
	admission with sepsis	34%	50%	0.24
	admission following surgery	30%	23%	0.39
	admission following trauma	**3%**	**5%**	**0.05**
Continued over page				

Illness course/ interventions prior to end-point achievement	median (IQR) Peak SOFA score	9 (8-12.5)	8 (5-11)	0.27
	% intubated	97%	85%	0.88
	% with central venous catheter	97%	85%	0.70
	% receiving renal replacement therapy	42%	39%	0.37
	% with tracheostomy	9%	6%	0.77
	% with urinary catheter	100%	90%	0.35
	% receiving H_2 antagonist/proton pump inhibitor	100%	93%	0.14
	% receiving corticosteroids	30%	23%	0.58
	% receiving a blood transfusion	52%	44%	0.42
	% receiving total parenteral nutrition	27%	18%	0.26
	median (IQR) length of stay (prior to infection)	6(5-7)	5(3-8)	0.22

Immune profiling	**% with elevated T-regs**	**60%**	**24%**	**0.004**
	% with low CD88	**94%**	**62%**	**0.001**
	% with low HLA-DR	**85%**	**55%**	**0.006**

Table VI-11: Patient demographic and clinical factors amongst those with and without nosocomial infection.
Right hand column indicates p value for univariate analysis (regression for continuous/ordinal variables and phi coefficient for binary categorical variables).

Variable	P value	**Hazard Ratio (95% CI)**
Overall model P value	0.037	NA
Shock on admission	0.51	1.3 (0.6-3.2)
Admission with trauma	0.65	0.75 (0.2-2.6)
*Elevated T-regs	0.05	2.1 (1.0-4.2)
C5a-mediated neutrophil dysfunction	0.05	4 (1.0-19.1)
Monocyte deactivation	0.07	2.4 (0.92-6.5)

Table VI-12: Cox model for occurrence of nosocomial infection.
*Elevated T-regulatory cells were expressed as a time-dependent co-variate.

Section 6.03 Chapter summary and discussion

In Chapter 5, the mechanism underpinning C5a-mediated neutrophil dysfunction was determined. However, as discussed, the physiological relevance remained uncertain. Furthermore, although a relationship between neutrophil dysfunction and ventilator-associated pneumonia was identified in Chapters 3 and 4, the temporal relationship between these two entities required further investigation.

Chapter 6 addresses these two issues. C5a-mediated neutrophil dysfunction is shown to be common amongst critically ill patients, and tends to occur early in the course of admission to ICU. Although some patients experience resolution of this dysfunction during their ICU stay, many have persisting dysfunction up until they are discharged or develop a nosocomial infection. This chapter also demonstrates the utility of neutrophil surface CD88 as a marker for this dysfunction, revealing that those patients with low CD88 (and hence C5a-mediated dysfunction) have a 5-fold increased risk of developing nosocomial infection. Given the association between low CD88 expression and ventilator-associated pneumonia identified in Chapter 4, it is interesting to note that there infection itself did not seem to depress CD88 further. These two findings suggest that neutrophil dysfunction is a risk factor for nosocomial infection rather than simply an epiphenomenon.

As noted in the Introduction (Chapter 1), and in Chapter 3, it is unlikely that neutrophil dysfunction is the sole immune defect with relevance to nosocomial infection acquisition. Chapter 3 revealed the presence of monocyte deactivation in patients with suspected VAP. In Chapter 6 this finding is confirmed and

extended, demonstrating that many critically ill patients have low monocyte HLA-DR expression and that this is also a predictor of nosocomial infection.

Regulatory T-cells have been identified in sepsis as being elevated, at least as a proportion of total lymphocytes and/or CD4 positive lymphocytes (Venet et al. 2009). This finding has been extended by showing similar elevation in patients with non-septic critical illness, suggesting that increases in these immuno-suppressive cells is part of the stereo-typed 'CARS' response to systemic inflammatory insults. Again, in keeping with monocyte deactivation and neutrophil dysfunction, elevated levels of T-regs are a significant predictor of nosocomial infection.

It is, however, the combination of these three immune dysfunctions that brings about the most interesting finding in this chapter. Absence of any dysfunction appears to protect against nosocomial infection, although caution must be expressed given the small number of patients who had no dysfunction. Cumulative addition of immune dysfunctions seems to bring about an additive increase in the risk of acquiring infection, to the point where those patients with all three have a 75% risk of acquisition which is far above that seen in patients with severe neutropaenia following chemotherapy or bone marrow ablation (GIMEMA authors1991). That this risk appears to be independent of other factors previously mooted as risks, such as severity of illness, invasive devices and co-morbid conditions, as well as offering a mechanistically plausible role in causation is particularly exciting. The implications of this will be discussed in Chapter 7 (Discussion and Conclusions).

Article VII. Discussion and conclusions

Section 7.01 Inflammation and dysfunction

In the work presented in Chapter 3 it was shown that critically ill patients with suspected VAP had evidence of immune activation and inflammation. Peripheral blood neutrophils demonstrated increased expression of CD11b, although as table IV-2 (page 126) showed in the following chapter this was not accompanied by increased basal or induced activation of the same receptor. Similarly serum markers of neutrophil activation, HNE and MPO, were elevated in patients. Serum hypercytokinaemia was also present, with elevation shown in a broad range of cytokines, anaphylatoxins, chemotaxins and colony stimulating factors. Of note was the concurrent elevation of the anti-inflammatory molecule IL-10. Examination of neutrophil-epithelial interactions revealed increased pro-inflammatory responses induced by neutrophils from patients, suggesting that the cells are 'primed' *in-vivo*, and respond excessively to a bacterial insult, demonstrating functional consequences of the noted immune activation.

Across most plasma/ peripheral blood cell measures there was little to differentiate those patients with microbiologically confirmed VAP from those whose clinical diagnosis was not confirmed, although there were differences in CD16 expression and serum HNE levels. This is in keeping with the existing work on SIRS and sepsis, demonstrating that both sterile and infectious insults

induce systemic activation of the innate immune system (Sakamoto et al. 2010, McILwain et al. 2010, Miyaoka et al. 2005). VAP has some additional effects on neutrophil activation, although this does not appear to translate into increased stimulated responses to LPS when co-cultured with the A549 type II alveolar epithelial cell line. In striking contrast, sampling the pulmonary compartment revealed a profound inflammatory response across a range of measures and which was largely restricted to those patients with confirmed VAP, indeed the response was sufficiently discriminatory as to offer diagnostic potential (Conway Morris et al. 2010).

Despite this abundant evidence of immune activation and inflammation, there were marked deficiencies in key cell functions. Neutrophils from patients were unable to efficiently phagocytose, and released diminished levels of superoxide ions in response to classic neutrophil stimuli. Both these processes are crucial to neutrophils' anti-bacterial functions (Hayward et al. 1979, Winkelstein et al. 2000), and their deficiency is likely to render patients vulnerable to infection. Similar defects in phagocytosis were identified in neutrophils which had transmigrated into the alveolar space, refuting the hypothesis that neutrophil dysfunction in critical illness is an artifactual finding resulting from sampling the peripheral pool when the more active cells have migrated into inflamed tissues.

In the other cell type examined, monocytes, again there was evidence of diminished function with reduced production of TNF-α in response to LPS stimulation, again a deficiency which has been linked to increased risk of infection (Landelle et al. 2010).

The findings of hypercytokinaemia and complement activation have been found in multiple human studies in critical illness (Sakamoto et al. 2010, McILwain et al. 2010, Miyaoka et al. 2005), as well as in animal models of conditions such as sepsis (Safranek et al. 2006), pancreatitis (Dib et al. 2003) and burns (Liu et al. 2008). Similarly the neutrophil activation measures reported have been found in similar studies (Raoof et al. 2010, Ogle et al. 1985). The compartmentalised, pulmonary inflammation in confirmed VAP is also consistent with published reports (Millo et al. 2004), although the diagnostic potential is a novel finding.

IL-10 is a potent anti-inflammatory cytokine (Moore et al. 2001), which influences the functions of numerous immune cells. Alongside inhibitory effects on neutrophils (Sun et al. 2009), it has been implicated in the pathogenesis of monocyte deactivation (Sfeir et al. 2001a), polarisation of macrophages towards the M2 phenotype (Martinez et al. 2008) and extends its influence into the adaptive immune system by polarising T-cells towards a regulatory phenotype (Langier, Sade & Kivity 2010). Indeed elevated levels of IL-10 have been promoted as a key mediator, and indicator, of the CARS. The simultaneous elevation of IL-10 and the pro-inflammatory cytokines noted in the patient cohort reported here is therefore an expected finding, although it is notable that this appears to be largely restricted to the peripheral rather than pulmonary compartment. Several previous investigators have noted the simultaneous elevation of pro- and anti-inflammatory mediators, and suggested that CARS be

modified to mixed anti-inflammatory response syndrome (MARS) to emphasise this duality (Bone 1996, Osuchowski et al. 2006).

What is a novel observation is the co-occurrence of immune activation and dysfunction within the same cell type, in this situation the neutrophil with its failure of phagocytosis and ROS production but retained ability to inflame tissue and release lytic enzymes such as HNE. Whether there are functionally distinct sub-populations of neutrophils responsible for this dichotomous behaviour, or if the cells are truly concurrently hyper and hypofunctional remains to be determined. It is tempting to speculate that the intense pulmonary inflammation in VAP occurs precisely because of this dichotomous behaviour, with the dysfunctional neutrophils unable to clear invading organisms which multiply to the point where their (the microbes') level is sufficient to stimulate the release of chemokines and lytic enzymes, resulting in further recruitment of dysfunctional neutrophils and tissue damage and further worsening respiratory function.

This study of VAP benefits from having a well characterised cohort of patients, satisfying strict pre-defined criteria for clinically suspected VAP, and representative of an important group of patients seen in all ICUs. By sampling both the peripheral and pulmonary compartments it was possible to identify both similarities and contrasts between these two areas. Furthermore, the volunteers were closely matched to the patients in age and sex, so limiting potential bias by comparing with healthy donors who were much younger and fitter.

However several important caveats remain. Firstly, these patients were sampled at a single time point, namely at the time of clinical suspicion of VAP, and this was typically 8 days into their ICU admission. Whilst it seems plausible that the immune defects preceded the infection, given the lack of differentiation of those patients with and without confirmed VAP the study design precludes excluding the possibility that immune dysfunction is an epiphenomenon of ICU-AI. Similarly although the intense pulmonary cytokine levels may well be the response to infection, again it is not possible to rule out the alternative explanation that patients with pre-existing alveolar inflammation are prone to infections (Meduri et al. 1995). Finally, although the control group were age- and sex-matched, they were not mechanically ventilated. Therefore it is possible that ventilation or underlying co-morbidities may explain some of the neutrophil dysfunction demonstrated in patients.

The implications from Chapter 3 are that critical illness, at least later on in the intensive care stay, does not result from either a pure hyper-inflammatory state or a pure, secondary, hypo-inflammatory response. It should perhaps be better viewed as a complex and maladaptive state displaying features of both immune activation and suppression/dysfunction. A similar situation can be found in the coagulation system during disseminated intravascular coagulation (DIC), where systemic and inappropriate activation of the coagulation system results in both thrombotic impairment of perfusion and simultaneous consumptive coagulopathy and bleeding risk (Bick, Arun & Frenkel 1999).

Section 7.02 C5a-mediated neutrophil dysfunction

Following the discovery of simultaneous immune activation and neutrophil suppression, the next step was to try and identify the mechanism underpinning the dysfunction. Although a range of plausible hypothetical mechanisms were developed, based on the phagocytic pathway (Lee, Harrison & Grinstein 2003) and possibilities raised by previous studies (Muller Kobold et al. 2000, Rosenbloom et al. 1999, Alexander et al. 1978, Bjornson, Bjornson & Altemeier 1981), it ultimately transpired that only the C5a-based mechanism found any support in my patient data.

As noted in the Introduction, the major issue with exploring C5a in human critical illness is its rapid plasma clearance and consequent short half-life (Webster, Larsen & Henson 1982).. The binding of C5a to CD88 and its subsequent internalisation and down-regulation of surface expression has been well established (Chenoweth, Hugli 1978) and previous studies have shown diminished CD88 expression on neutrophils from critically ill patients (Furebring 2005). However prior to this study CD88 had not been used as a proxy measure for neutrophil C5a exposure, and there had been no previous demonstration of any relationship between CD88 and neutrophil functions such as phagocytosis. The negative correlation which exists between C3a (and to a lesser extent C5a) and phagocytosis, alongside the failure of any other cytokine or cell surface markers examined to correlate, further strengthened the case for the defect in phagocytosis being associated with complement activation.

It is important to note that C5a is not the only molecule known to influence the expression of CD88. Data from this study demonstrated that interferon-gamma could increase CD88 expression (Figure V-20, page 163), whilst Furebring and colleagues showed TNF-α and IL-8 could diminish its expression (Furebring et al. 2006). However TNF-α and IL-8 only reduced CD88 expression when applied at levels two to three orders of magnitude higher than those measured in the patients in the studies presented here (table III-3 page 109 and tables VI-3,4 page 189), whilst the concentration of interferon gamma used (5ng/ml) is higher than that seen in previous studies of severe sepsis and organ failure (Pinksy *et al.* 1993). Therefore, although we can be fairly confident that CD88 expression is largely determined by *in-vivo* C5a exposure, we cannot definitively rule out the influence of other inflammatory molecules which could confound the association seen and reduce the usefulness of CD88 in this context.

C5a has classically been viewed as a pro-inflammatory molecule, with positive effects on neutrophil priming (Bajaj et al. 1992), chemotaxis (Snyderman et al. 1975), neutrophil recruitment (Snyderman, Phillips & Mergenhagen 1971) and degranulation (Bender, McPhail & Van Epps 1983) . However these effects have largely been demonstrated at relatively low concentrations (10pM-1nM), and following relatively short durations of exposure (seconds to minutes). Animal models, and *in-vitro* work, had identified C5a in the higher concentrations seen in sepsis as a mediator of neutrophil dysfunction, including both impaired phagocytosis and ROS productions (Huber-Lang et al. 2002b). These findings had been identified in both septic (Huber-Lang et al. 2002b) and sterile (Flierl et al. 2008a) animal models. The findings from patients in this

work was the first time this effect had been demonstrated in humans, and is an important step in translating the work from animal studies into clinically useful research.

Developing the association between markers of C5a exposure and neutrophil dysfunction shown in Chapter 4 into evidence for a causal relationship required exploration of the intra-cellular pathways by which any effect may be mediated. As the data in Chapter 5 demonstrated, recombinant C5a applied to healthy donor neutrophils was able to induce not only a defect in phagocytosis (figure V-1, page 144) but also replicate the correlation between CD88 and phagocytosis (figure V-2, page 145). The effect of C5a on phagocytosis was prevented by blocking it's binding to CD88, so providing the first step in the mechanistic pathway.

Ligation of CD88, in common with other heptahelical receptors, results in signal transduction via the coupled G-proteins and their down-stream effector molecules. CD88 is coupled to the same G-protein alpha sub-unit ($G_{\alpha i2}$) as the fMLP receptor FMLR, and ligation of this second receptor with equimolar concentrations of fMLP induced the same defect in phagocytosis as C5a (figure V-3 page 146).

The down-stream pathways activated by C5a (and fMLP) include the adenylate cyclase/cAMP/protein kinase A (PKA) pathway (Tsu, Allen & Wong 1995), PI3Ks (Konrad et al. 2008) and protein kinase C (PKC) pathway (Wrann et al.

2007b). Beta-adrenoceptor agonists such as isoproterenol are known to impair phagocytosis, and this is facilitated by accumulation of cAMP (Ignarro, Lint & George 1974),. Whilst this effect of beta agonists was reproduced in the current study (figure V-4 page 148) and was prevented by the inhibition of adenylate cyclase, neither C5a nor fMLP appeared to mediate their inhibition of phagocytosis by this route (figures V-5 to V-7 pages 148-49).

PI3K was the next target molecule examined, given its known effects on actin polymerisation and phagocytosis (Pinho et al. 2007, Cox et al. 2001). Pre-treatment of neutrophils with two separate inhibitors of PI3K, IC87114 and wortmannin, was able to prevent C5a's effects on phagocytosis (figure V-8, V-9, page 151-2). Given that PI3K has generally been seen as a positive promoter of phagocytosis (Cox et al. 1999), this finding was initially rather counter-intuitive. Two possibilities presented themselves, either the effects seen could be due effects of these two inhibitors on non-PI3K enzymes (Davies et al. 2000) or that differential involvement of PI3K isoforms was involved, which would not be clear from the use of non-selective inhibitors such as LY294002 and wortmannin. Given that both non-selective inhibitors produced the same effect it seemed less likely that this was due to non-PI3K inhibition. Furthermore the effect of LY294002 persisted well below the reported IC_{50} for PI3Kγ (figure V-9, page 151) also argued against this and in favour of an isoform specific effect.

The two predominant types of class I PI3Ks found in leukocytes, including neutrophils, are the gamma (γ) and delta (δ) isoforms (Rommel, Camps & Ji 2007) both of which are activated by C5a (Konrad et al. 2008). The

experimental data suggest that the effects of C5a on phagocytosis are mediated by the δ form (figure V-10, V-11, pages 153-4). The ability of AS605240 to inhibit protein kinase B (PKB) phosphorylation (figure V-12, page 156) demonstrated its functionality, whilst concentrations of IC87114 that were able to fully inhibit C5a-mediated phagocytic dysfunction did not fully inhibit PKB phosphorylation (Figures V-11, V-12, pages 154-56). This, alongside published evaluations of the specificities of these two inhibitors (Ferrandi et al. 2007, Smith et al. 2007, Knight et al. 2006) and the functioning of LY294002 at low concentrations supports the contention that the effect is indeed specific to the δ isoform. Specific activation of the class IA PI3Ks, using 740 Y-P, was able to induce a similar defect in phagocytosis to that achieved by C5a (figure V-13, page 157).

The inhibition of RhoA downstream of PI3Kδ (figure V-14, page 158) is consistent with work by other groups on the effects of this isoform in cell lines and primary macrophages (Papakonstanti et al. 2008, Papakonstanti, Ridley & Vanhaesebroeck 2007). Intriguingly, high intracellular concentrations of cAMP also impair phagocytosis by inhibiting RhoA (Kamanova et al. 2008), suggesting a common final pathway through which both C5a and cAMP may work despite disparate up-stream mechanisms (see above).

Actin polymerisation and reorganisation is the final step in the process of phagocytosis, and in the complement-mediated situation this is dependent on RhoA activity (Caron, Hall 1998). 1C5a impaired actin polymerisation, and this effect could be prevented by both pan PI3K inhibition with LY294002, and also

delta-specific inhibition with IC87114. The proposed pathway from C5a ligation of CD88 through to impaired phagocytosis is shown in figure VII-1 below.

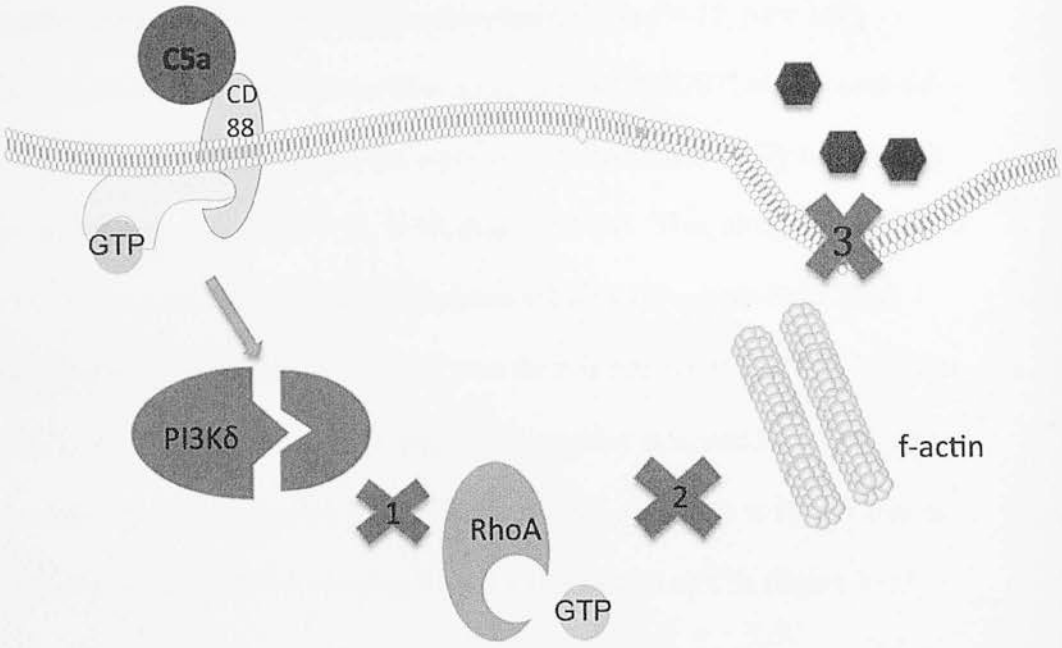


Figure VII-1: Schematic representation of C5a's proposed effects on neutrophil phagocytosis.
Reading sequentially from the top left - C5a engages its G-coupled receptor CD88 on the neutrophil surface. This activation activates (green arrow) the delta isoform of phosphoinositide-3-kinase (PI3Kδ). Activation of PI3Kδ inhibits RhoA (red cross marked 1). Inhibition of RhoA results in failure to polymerise actin (red cross marked 2). As actin polymerisation is required for effective phagolysosome formation (red cross marked 3), the result is impaired phagocytosis of pathogens/pathogenic particles (represented by purple hexagons).

Granulocyte-macrophage colony stimulating factor (GM-CSF) was first identified as a growth factor for myeloid cells, facilitating proliferation and differentiation (Stanley et al. 1976). However it also has recognised cytokine-

like effects on immune cell priming, reactive oxygen species production and transmigration, as well as impacts on pulmonary surfactant production and alveolar barrier function (reviewed in Hamilton, 2002) A study in non-neutropenic sepsis suggested that GM-CSF was able to improve neutrophil phagocytic capacity (Presneill et al. 2002), and with serum levels being found to be low in patients with clinical VAP (Tables III-3 and III-5, pages 109-10), it appeared to be a potentially useful therapeutic option. As this study demonstrated, GM-CSF was able to restore phagocytosis by healthy neutrophils following *in-vitro* exposure to C5a.

The mechanism by which GM-CSF acts to restore phagocytotic effectiveness following exposure to C5a remains obscure. However it is clear from figures V-20,21 (page 163) that it does not function by restoring CD88 expression. In keeping with the suggested role of reduced CD88 as a marker of C5a exposure rather than it acting as a phagocytic receptor, restoring CD88 expression through the use of interferon gamma did not resurrect phagocytosis (figure V-20, 21). Furthermore, the addition of the PI3Kδ inhibitor IC87114 *after* exposure to C5a did not restore phagocytosis in contrast to its ability to block the effects of C5a when cells were treated prior to exposure. GM-CSF is, however, able to reverse the defects in RhoA signalling (Figure V-18, page 161) and restore actin polymerisation (Figure V-19, page 162).

Therefore it seems probable that GM-CSF acts downstream of CD88/PI3Kdelta (see figure 7-1 above). The pathway from GM-CSF ligation of its receptor to the restoration of RhoA activity requires further study, but published data suggests

this may be mediated via the lyn/src-kinases (which can in turn activate RhoA (Shi et al. 2006a, Yousefi et al. 1996). Work on this area is currently ongoing.

The inhibitory effects of C5a on neutrophil function are not restricted to phagocytosis. C5a-treated neutrophils demonstrated significant reductions in bactericidal function against *Pseudomonas aeruginosa* (figure V-23, page 165), and also impaired transmigration towards fMLP (figure V-24, page 166). The impairment of transmigration is an interesting finding as, in contrast to phagocytosis, it did not appear to be mediated by PI3Kδ (Figure V-25, page 167), indeed PI3Kδ itself was crucial to fMLP-stimulated transmigration (Figure V-25).

Although the finding of PI3Kδ inhibition preventing transmigration is consistent with the existing literature (Sadhu et al. 2003), it is surprising given the known dependence of transmigration on RhoA (Worthylake et al. 2001). It is notable that Papakonstanti's study (Papakonstanti et al. 2008) showed PI3Kδ inhibition prevented transmigration despite the simultaneous and expected rise in RhoA. It appears that transmigration relies on a delicate balance of intracellular RhoA levels. Whilst inhibition impairs transmigration (Worthylake et al. 2001), heightened levels can impair rather than promote transmigration (Ridley, Comoglio & Hall 1995). In addition, other investigators report a threshold effect of RhoA, where only high concentrations of RhoA inhibitor impaired transmigration (Nobes, Hall 1999). Furthermore, the effects of PI3Kd inhibition on neutrophil transmigration do not appear to act through impairment of actin

polymerization (Sadhu et al. 2003). In contrast, the work above demonstrated that actin polymerisation was central to the effects of PI3Kd inhibition on phagocytosis. It therefore seems plausible that PI3Kd inhibition restores sufficient RhoA to allow efficient phagolysosome formation and phagocytosis in C5a-treated neutrophils, but that levels of RhoA are insufficient to restore transmigration (and/or that in dysfunctional neutrophils restoration of transmigration requires PI3Kd/RhoA-independent pathways). As noted above, elevated cAMP impairs phagocytosis by impairing RhoA activation (Kamanova et al. 2008), however elevated cAMP does not appear to inhibit transmigration (Daniels et al. 1993), again suggesting that C5a's inhibition of transmigration may work via an alternative mechanism(s).

Interestingly, the dissociated effects of PI3Kd inhibition on phagocytosis and transmigration in dysfunctional, C5a-treated neutrophils could explain why PI3K inhibitors have not proven beneficial in sepsis (Wrann et al. 2007a). Certainly these findings do not suggest a therapeutic place for PI3Kd or RhoA inhibitors for prevention of infection in critically ill patients, in contrast to the potential for GM-CSF.

The pathway by which recombinant human C5a is able to impair phagocytosis appears relatively well delineated in the above experiments, however the key to translating this into a clinically useful finding is seeing whether the neutrophils from critically ill patients demonstrate similar features. Similar to neutrophils from patients with VAP (Chapter 3), those recruited at an earlier time-point, prior to acquiring nosocomial infection, displayed impaired phagocytosis,

diminished CD88 expression and a strong correlation between these two measures (Figures V-26, 27, pages 168-9). Although CD88 has been shown, *in-vitro*, to be depressed by IL-6 and TNF-a as well as C5a (Furebring et al. 2006), these effects occurred only at very high concentrations, far above those seen even in sepsis (Table III-3, page 109). In contrast C5a is able to produce a similar diminution in CD88 to that seen in patients at concentrations found in sepsis (Solomkin et al. 1981).

Further evidence of C5a-mediated phagocytic dysfunction in patient neutrophils comes from the demonstration of impaired RhoA activation in response to zymosan (Figure V-28, page 170), coupled with a similarly impaired actin polymerisation (Figure V-29, page 171). GM-CSF, applied *ex-vivo*, was able to resurrect phagocytosis. Defective actin polymerisation responses have been noted in neutrophils from patients with burns (Vindenes, Bjerknes 1997). RhoA down-regulation has been noted in septic myocardium following prolonged cGMP exposure (Sauzeau et al. 2000, Buckley, Singer & Clapp 2006), although it had not previously been identified in critically ill humans generally or neutrophils specifically.

The ability of a pro-inflammatory molecule to provoke a dysfunctional state begs the question as to how such an apparently maladaptive situation evolved. It may be simply that the sorts of insults that provoke the massive, systemic release of complement would normally be unsurvivable without modern intensive care, and therefore the maladaptive processes encountered in such a situation would be

unable to apply any selective pressure. It may also be an unfortunate side effect of attempted counter-regulatory mechanisms. For instance RhoA appears to play a dual role in neutrophils, inhibiting NFkappaB-dependent TNF-α release in the resting state but promoting the same pathway in the LPS-activated state (Fessler et al. 2007). It may be that the effects of C5a are an attempt by the cell to inhibit RhoA, so down-regulating pro-inflammatory molecule production but resulting in the unwanted side effect of impaired phagocytosis.

As all the work on the mechanism by which C5a inhibits phagocytosis was conducted in primary human neutrophils, and included findings involving neutrophils from critically ill patients, it is likely that this represents findings of direct relevance to the clinical situation. The significant differences between human and murine immune systems in general (Mestas, Hughes 2004) and signalling pathways specifically (Condliffe et al. 2005) suggest that findings from animal models may not be directly transferable to humans. Similarly the use of immortalised cell lines, even those derived from human cells, can be questioned as different isoforms of key enzymes such as PI3K can predominate compared to primary cells (Papakonstanti et al. 2008).

However, although the work was conducted in primary human cells, a potential issue with this study relates to the relevance of the assay used to assess phagocytosis. Zymosan particles are derived from yeast (*Saccharomyces cerevisiae*) and are large compared to other phagocytic targets such as bacteria. Furthermore the use of cells adhered onto tissue culture plastic in tissue culture

medium may not fully reflect the physiological and pathophysiological situations found in healthy and diseased humans. As such there remained a risk that the observations reflect *in vitro* artefacts specific to the model used. This issue was addressed in the study reported in Chapter 6, discussed below, into the ability of C5a-mediated dysfunction to predict risk of nosocomial infection acquisition.

The identification of C5a as a key mediator of neutrophil dysfunction has several important implications. C5a is released early in critical illness (Böttiger et al. 2002, Fosse et al. 1998), suggesting that dysfunction may also occur earlier than has been proposed for a classical CARS response (Schefold et al. 2008). Complement activation occurs in a variety of diseases that precipitate critical illness and this may explain the apparently ubiquitous nature of the increased risk of nosocomial infection amongst patients admitted to the ICU (Vincent 2003). What stimulates the systemic, apparently uncontrolled activation of complement and release of C5a in critical illness remains uncertain. A variety of complement activation mechanisms may be present in critical illness, including coagulation (Amara et al. 2010a), human neutrophil elastase (HNE)-mediated cleavage of IgG to form F(ab') portions (Fumia et al. 2008), HNE itself (Vogt 2000) and systemic release and circulation of bacterial peptides (Thorgersen et al. 2009). Whether systemic activation occurs simply because these activators are released in large quantities, or whether there are underlying defects in the complement regulatory mechanisms, remains to be determined.

The strong correlation between neutrophil CD88 expression and phagocytosis suggests that CD88 may be able to act as a biomarker for dysfunction, which

would aid the clinical utility of this finding as it would remove the need for laborious and technically challenging separation of neutrophils and subsequent phagocytosis assays.

GM-CSF offers a potentially exciting therapeutic option. To date there have been several small trials of GM-CSF in non-neutropaenic sepsis (Meisel et al. 2009, Presneill et al. 2002, Orozco et al. 2006, Rosenbloom et al. 2005), of which only one (the smallest) directed therapy towards patients with identified immune dysfunction, i.e. monocyte deactivation (Meisel et al. 2009). Presneill and colleagues demonstrated GM-CSF-mediated improvements in phagocytosis (Presneill et al. 2002), and encouragingly although all studies showed the expected increase in blood neutrophil counts none showed any evidence of harm. Indeed GM-CSF improved rather than worsened pulmonary function in patients with ARDS, possibly through its effects on surfactant production (Pelaez et al. 2004). Existing trials have been too small to show convincing effects on outcomes, however the study by Orozco and colleagues did indicate a reduction in intra-abdominal infections.

GM-CSF may not be the only potential therapy, although its pluripotent effects and effective action in the presence of established dysfunction make it an attractive option. Several small molecule CD88 antagonists exist, including PMX53 that has undergone human trials (Vergunst et al. 2007) and appears safe and well tolerated in inflammatory disease. Its apparent safety not withstanding, PMX53 did not have a significant effect on rheumatoid disease activity, and furthermore blocking CD88 may also prevent beneficial functions of C5a on

neutrophil recruitment and bacterial killing. Studies of CD88 knockout mice show impaired clearance of the important nosocomial pathogen *Pseudomonas* (Hopken et al. 1996), so making this potential therapeutic avenue less attractive. Pre-clinical studies have shown anti-C5a antibodies to be effective at preventing sepsis induced immune dysfunction (Huber-Lang et al. 2001), however they have not yet been demonstrated to be effective in a treatment situation and indeed have yet to be translated into a humanised form suitable for the clinical environment. C5a could also be removed from the circulation by extra-corporeal absorption, a technique which already has been demonstrated in humans and which results in a restoration of CD88 expression (Schefold et al. 2007). With the recent beneficial findings from extracorporeal LPS absorption (Cruz et al. 2009), one could envisage a combined absorber that may be effective, although extracorporeal circulation brings its own risks (Zimpfer et al. 2006).

It is important to note that C5a is not the only mediator of neutrophil dysfunction identified. The BALF from patients contains a potent inhibitor(s) of phagocytic function that is distinct from C5a, and importantly this inhibitor was not found in BALF from matched volunteers, suggesting that it is specific to the inflamed lungs found in critical illness. Although this study was able to exclude C5a as the causative factor, the actual nature of the inhibitor(s) and its mechanism of action remain obscure. However the presence of a specific pulmonary inhibitor may explain the high prevalence of pulmonary infections amongst critically ill patients (Vincent et al. 1995, Vincent et al. 2009)

Section 7.03 Clinical implications of C5a-mediated neutrophil dysfunction and other immune dysfunctions

Having identified C5a as a key mediator of neutrophil dysfunction and defined the mechanism by which it inhibits phagocytosis, the question arises of clinical relevance and time course. In the study of patients with suspected VAP the samples were taken at the time of clinical suspicion, which as table III-1 (page 98) shows was typically 8 days after admission to ICU, and therefore it was not possible to determine whether C5a-mediated dysfunction preceded, or was a consequence of, nosocomial infection. In addition, although immune depression is suggested to be a late phenomenon in critical illness (Hotchkiss, Karl 2003, Schefold et al. 2008), C5a release occurs early (Fosse et al. 1998) and therefore if the hypothesis is correct dysfunction should occur similarly early.

The correlation between CD88 and impaired phagocytosis, the hallmark of C5a-mediated neutrophil dysfunction in humans, was found in samples taken early in the patient's ICU stay (Figure V-26, page 168), importantly before any ICU-acquired infection had occurred. Figures VI-2 and VI-3 (pages 180-1) demonstrate that neutrophil CD88 was below levels seen in healthy volunteers in the majority of patients at study admission (i.e. <48 hours after ICU admission), with counts tending to fall further during admission suggesting ongoing complement activation. Interestingly when C3a (a proxy measure of C5a release) was examined there was a marked dichotomy amongst patients with and without dysfunction - those with dysfunction showed increases in C3a concentrations during their ICU stay, whilst those without dysfunction showed a trend towards decreasing C3a (Figure VI-5, page 187). Similarly peak C3a

levels were higher in the group with dysfunction (table VI-4, page 188). These findings are suggestive of on-going complement activation in the patients with neutrophil dysfunction/low CD88. Notably, there was no such dichotomy between the groups in the case of other inflammatory mediators (table VI-3 and VI-4, page 188).

Patients with neutrophil dysfunction had a considerably higher risk of nosocomial infection (RR 5.4 95% CI 1.4-21.0). As this effect could, potentially, be biased by length of stay it was reassuring that the time-series analysis showed a significant difference between the two groups, with the dysfunction group showing a steady increase in infections over their stay whilst those without dysfunction remained relatively resistant to infection even if they remained in the ICU for some time (figure VI-6, page 190). This effect remained significant if corrected for demographic and clinical differences between those with and without dysfunction in a Cox model (table VI-5, page 189).

C5a can inhibit a range of neutrophil functions including transmigration and reactive oxygen species production (Huber-Lang et al. 2002b, Blackwood et al. 1996). Therefore one cannot be certain that the phagocytic defect specifically leads to the increased risk of nosocomial infection observed. Indeed it is likely that multiple defects act synergistically to produce the immunoparalysis that appears to characterise neutrophils in such severely ill patients.

Although steps were taken to reduce the risk of simply detecting an epiphenomenon of infection, by censoring data for 2 days prior to diagnosis of

infection, there remains a possibility that sub-clinical infection was responsible for the differences seen in CD88 expression. However, were this to be the case, one might expect to see lower CD88 expression in patients admitted with infections (i.e. sepsis) compared to sterile insults, or reductions in CD88 after the acquisition of nosocomial infection. In fact no such effects were seen (Figures VI-7, VI-8, page 191), reducing the likelihood that the effect was an epiphenomena of infection.

It is interesting to note that patients admitted to the ICU with hospital-acquired infections universally demonstrated CD88 levels below the cut-off for dysfunction in contrast to patients with community acquired-infections (Figure VI-9, page 192). This suggests that C5a-mediated neutrophil dysfunction may not be restricted to patients in the ICU, and may be present in those general ward patients at risk of nosocomial infection. This study was not designed to answer this question, but it raises interesting questions for future study.

Neutrophils are only one of a number of immune cells that have been implicated in critical-illness related failure (see section 1.05, Chapter I). Within the circulating compartment, both deactivated monocytes and elevated levels of T-regs have been demonstrated (Venet et al. 2009, Volk et al. 1996). Monocyte deactivation is the best-studied example of immune dysfunction in critical illness, and has been linked to nosocomial infections (Landelle et al. 2010, Cheron et al. 2010) although this finding is not universal (Lukaszewicz et al. 2009). High levels of T-regs, on the other hand, had not been linked to this outcome before the current study.

This work confirmed the results of other groups, identifying evidence of monocyte deactivation using HLA-DR expression (Höflich et al. 2002). However in contrast to some published evidence (Monneret et al. 2006), monocyte deactivation was found to occur early on in the ICU stay in a manner similar to neutrophil dysfunction. Regulatory T-cells were also found to make up an increased proportion of the patients' T-cells, although the peak for this tended to occur at a later time point than the other dysfunctions (Figure VI-11, page 196), and displayed a greater degree of variability during the course of the ICU stay (Figure VI-11).

Both monocyte deactivation and elevated levels of regulatory T-cells showed an association with the acquisition of nosocomial infection, although monocytes lost their significance in the Cox model (Table VI-12, page 208). Interestingly there appeared to be an additive effect of immune dysfunction on the risk of nosocomial infection, with a greater burden of immune dysfunction predicting a progressively greater risk of acquiring infection (Table VI-10, page 204).

This study has a number of strengths. It involved assessment of several distinct mechanisms of immune dysfunction, and allowed for dynamic changes in immune cell function rather than relying on a single time point. Immuno-phenotyping in critical illness is a new field, however it is encouraging that two out of three cut-off values for dichotomising patients into 'dysfunction' or 'no dysfunction', derived from preliminary work, performed well at that cut-off and did not require further post-hoc adjustment which could be a source of bias. The

infections had to meet rigorous, reproducible criteria and any cases that did not meet these criteria were reviewed by experienced clinicians who were blinded to the immune cell data.

The sample size was relatively small, however it was close to the intended size derived from the power calculation. As with any observational study one cannot be certain that any associations are causative, however steps were taken to minimise the risk of picking up epiphenomenal changes associated with infection and therefore one can be reasonably certain that the changes in immune function preceded the acquisition of infection. Furthermore, the supposition that immune dysfunction is causally linked to the acquisition of nosocomial infection is biologically plausible. Although residual confounding from unmeasured variables cannot be ruled out, many of the risk factors previously mooted for nosocomial infection have been examined (Vincent 2003). However it is important to note that most clinical risk factors have been analysed in a binary (i.e. present/absent) fashion, which does not take into account the duration of their presence (e.g. number of days of mechanical ventilation or central venous access). The relatively small number of infection events precluded building cox models with multiple time-dependent variables, which would be required to disentangle the effects of length of stay from duration of specific clinical risk factors. These considerations will inform the design of the planned validation study which will seek to replicate the results demonstrated above.

Several previous studies have demonstrated elevated levels of T-regs in patients with sepsis (Venet et al. 2009, Venet et al. 2008), and recent animal and *in-vitro*

work has shown T-regs mediating impaired T-cell proliferative responses in this disease (Venet et al. 2009). Although such impaired proliferative responses have been known for some time (Hotchkiss, Karl 2003, Mahlknecht et al. 1996), the mechanism(s) had remained uncertain. The current study extends the findings of elevated T-reg cells to critically ill patients without sepsis, suggesting that their elevation is part of a stereotyped response to systemic inflammation rather than a specific response to severe infection.

This is the first study to examine several different markers of immune dysfunction simultaneously, and to demonstrate an additive effect when it comes to predicting nosocomial infection. It remains a distinct possibility that the effects are not simply additive, but may indeed be synergistic, for instance T-regs may induce alternative macrophage activation (Tiemessen et al. 2007) and so possibly alter monocyte function in a similar manner. T-regs also alter neutrophil functions *in-vitro* (Lewkowicz et al. 2006). The investigation of potential interactions will be the subject of future studies.

It is interesting to speculate why this study did not show significant effects of demographic and clinical factors that have been previously linked to nosocomial infection (Vincent 2003). It is important to note that although there are a variety of acknowledged risk factors, including severity of illness, intubation, total parenteral nutrition and tracheostomy (Pratikaki et al. 2008, Nourdine et al. 1999, Crowe et al. 1998, Brook et al. 2000), the various studies showing these effects often produce different combinations of factors and show reasonable disagreement between such combinations (Pratikaki et al. 2008, Sugerman et al.

1997, Moro, Viganò & Cozzi Lepri 1994). Moreover, many studies do not adequately account for the relationship between interventions such as tracheostomy, blood transfusion and total parenteral nutrition, and length of stay (i.e. duration of risk exposure), in that the longer one has to remain in ICU the more likely one is to receive one of these interventions (Crowe et al. 1998, Levine et al. 2010), with the added confounder that those acquiring nosocomial infection subsequently tend to stay on ICU longer (Beyersmann et al. 2008). Many epidemiological studies of infection in ICU include all comers, whereas in this study we deliberately recruited a group who were thought to be at high risk. All the patients had some form of invasive device in place, be it endotracheal tube, central venous catheter or haemofiltration line, to facilitate the organ support that was an entry criterion. With a median APACHE II score of 22 (IQR 18-28), these patients were a sicker subset of all patients admitted to the unit in question (Scottish Intensive Care Society Audit Group, 2009). As such it is likely that the effects of other factors on nosocomial infection had been reduced, allowing a clearer view of the effect of immune dysfunction. This selectivity may have some limiting effect on wider applicability, however it is likely that any therapeutic or preventative interventions for nosocomial infections would be concentrated on patients such as those who entered this study.

The sampling schedule was derived from pragmatic considerations, balancing the desire for as complete a picture as possible of immune cell changes with the feasibility of collection and analysis of fresh blood samples. Prior to undertaking this study the time course of immune dysfunction was unclear, so the sampling schedule was based on reasonable supposition as to the likely timing of any

defects. These two factors mean that we have little data on the very early phases of hospital and ICU admission of the patients, whilst not having sampling beyond the acquistion of first infection or discharge from the unit. These factors will help inform the design of the planned validation cohort study arising from this work.

The immunophenotyping we performed used basic, three colour flow cytometry. As such this work could be readily translated into clinical practice in any hospital with access to a flow cytometer. The suggestion that intracellular FOX-P3 staining can be replaced by surface CD127 negativity (Venet et al. 2009) would further simplify matters and speed up assessment, giving a 'read out' of immune status within 30 minutes of sample arrival. The reliability and variance of the measures of immune function within a single patient sample have not been addressed in this study, but such assessments will be necessary before any clinically valid immuno-phenotyping assay could be developed.

The potential uses of such immune profiling are several. By enabling risk stratification of patients it could allow targeted immuno-stimulatory interventions (Meisel et al. 2009), and allow for more carefully designed trials of such interventions. Another use may be to identify those at greatest risk and direct prophylactic measures at them, so minimising the use of expensive and potentially harmful interventions amongst patients who are unlikely to benefit. Finally it may help answer some of the questions around reductions in nosocomial infections, allowing effective comparisons between and within units

by correcting for baseline risk prior to any patient safety/quality improvement programmes (Conway Morris, A 2011 b).

Section 7.04 Future work

This work provokes as many questions as it answers, and suggests a range of avenues for future work. On the mechanistic level, there is the question of how GM-CSF is able to restore RhoA activation and phagocytosis, as well as its effects on transmigration. Second is the possibility of translating the GM-CSF therapy from the lab to the patient, testing whether it can restore phagocytic function in the first instance and also whether doing so will reduce the risks of acquiring nosocomial infection. With GM-CSF also having effects on monocyte deactivation, which co-exists with neutrophil dysfunction, it may have a key role to play in the management of immune suppression in the ICU. In order to target therapy at those patients most likely to benefit, CD88 shows promise as a biomarker, though the finding of its association with nosocomial infection requires confirmation in an independent cohort. Finally, several areas that have received less focus in this thesis may also prove fruitful avenues for exploration. The finding of intense pulmonary inflammation in microbiologically confirmed VAP has important implications for potential diagnostic biomarkers, and this background formed the basis of a study that has recently been funded (HICF 0510-078). The nature of the pulmonary inhibitor of phagocytosis requires further clarification, as well as an evaluation of potential therapies to counteract its effects. Finally the work on CD88 may have implications outside the ICU, amongst general ward patients and should the validation study prove positive it would be the next, logical place to take this work.

Section 7.05 Conclusions

This study has demonstrated that critical illness is characterised by a complex inflammatory state, with features of both immune hyper-activation and suppression present simultaneously and within single cell types. This remarkable duality is illustrated by the ability of a pro-inflammatory molecule, C5a, to drive the neutrophil dysfunction seen in critical illness. This finding from patients was developed by *in-vitro* manipulation of healthy cells to demonstrate a specific intracellular pathway by which the phagocytic machinery can be inhibited, before returning to patient neutrophils to demonstrate similar mechanistic features. In GM-CSF a potential treatment has been identified. Finally, this work has demonstrated that C5a-mediated neutrophil dysfunction precedes nosocomial infection and acts as marker of risk for such an adverse outcome. The use of simple cell surface marker quantification by flow cytometry raises the potential for this to be used to identify clinically useful biomarkers of dysfunction, allowing the targeting of therapies such as GM-CSF. Neutrophil dysfunction does not exist in isolation, co-occurring with at least two further immune failures i.e. hypoactive monocytes and excessive levels of regulatory T-cells. These cellular dysfunctional states can also be readily phenotyped using simple tools, and this raises the possibility of effective immune system monitoring to complement the monitoring of other organ systems that is undertaken in the intensive care unit.

The findings of profound immune suppression provide the pathophysiological rationale for immuno-stimulatory therapies, whilst the identification of specific pathways and agents that modulate these pathways suggests what such immuno-

stimulatory agents may be. The era of immuno-suppressive therapies for sepsis and SIRS has now largely passed, with few if any effective treatments remaining from this concept. Perhaps now the time has come to look at and treat the other side of immune (dys)function in critical illness.

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Publications arising from this work

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