Does GM-CSF restore effective neutrophil function in critically ill patients?

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Abstract

Nosocomial infection is an increasing problem worldwide associated with significant morbidity and mortality in addition to heightened healthcare costs. The problem is even greater on the intensive care unit (ICU) where up to 20 of patients develop a secondary infection during their admission. In an era of increasing antibiotic resistance alternative strategies to prevent nosocomial infection must be sought.

The intensive care population are recognised to be at high risk of developing immune dysfunction during their critical illness and this has been shown to be associated with an increased risk of the development of ICU acquired infection (ICUAI). The neutrophil, in particular, is key in terms of the host response to bacterial and fungal infections and impairments in neutrophil function have been demonstrated in critically ill patients.

Granulocyte-macrophage colony-stimulating factor has been shown to improve the phagocytic function of impaired neutrophils ex-vivo and has previously been demonstrated to restore immune competent levels of monocyte HLA-DR expression in critically ill patients.

If GM-CSF were demonstrated to restore neutrophil phagocytic function in critically ill patients in whom its known to be impaired it may have a role in preventing the development of ICUAI.

Our initial study sought to validate neutrophil CD88 expression as a surrogate marker for phagocytic function. The dose finding study which followed aimed to determine the optimum dose and duration of GM-CSF to be carried forward to a randomised controlled trial. Finally, the randomised controlled trial sought to investigate the hypothesis that GM-CSF could restore effective neutrophil function in critically ill patients.

While no significant difference was seen in our primary endpoint of neutrophil phagocytic capacity, on day 2 following administration of GM-CSF, we believe a small but true biological effect was observed suggesting further study is warranted to investigate whether GM-CSF could reduce the risk of ICUAI.

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I commenced work on these studies in October 2011 writing the trial protocol, securing regulatory approval and completing procurement of the IMP. With the support of members of the trial team I oversaw the set-up of the study at each site. I completed all aspects of recruitment and follow-up of participants within the assay confirmation and dose finding studies at the Newcastle sites. I completed all research laboratory analyses for all patients recruited during these phases of the trial. During the randomised controlled trial, I completed all of the laboratory analyses for the first seven patients following which, I returned to full time clinical work in February 2014. Between February 2014 and June 2014 I continued to contribute to laboratory work at certain times during weekends. Subsequent completion of this trial relied on the commitment and dedication of my colleagues who took on responsibility for the clinical and laboratory aspects of the study following my departure in February 2014. I am immensely grateful to the each of the following people for their contributions to this work.

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Abbreviation	Full Wording
AAA	Abdominal aortic aneurysm
Ab/cell	Antibodies per cell
ACCP	American College of Chest Physicians
ACS	Assay confirmation study
AE	Adverse event
AKI	Acute kidney injury
ALI	Acute lung injury
ALD	Alcoholic liver disease
ALP	Alkaline phophatase
ALT	Alanine aminotransferase
ANC	Absolute neutrophil count
ANCOVA	Analysis of covariance
APACHE	Acute Physiology and Chronic Health Evaluation
APC	Allophycocyanin
ARDS	Adult respiratory distress syndrome
AST	Aspartate aminotransferase
AVR	Aortic valve replacement
BD	Becton Dickinson
BMI	Body mass index
BP	Blood pressure
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CAP	Community-acquired pneumonia
CARS	Compensatory anti-inflammatory response syndrome
CI	Chief investigator

CLP	Caecal ligation and puncture
CO_2	Carbon dioxide
CONSORT	Consolidated Standard of Reporting Trials
COPD	Chronic obstructive pulmonary disease
CRF	Case report form
CTIMP	Clinical trial of investigational medicinal product
CTU	Clinical Trials Unit
CVVH	Continuous veno-venous haemofiltration
DAMPS	Damage associated molecular patterns
DFS	Dose finding study
dL	Decilitre
DMSC	Data Monitoring and Safety Committee
DNA	Deoxyribonucleic acid
ECG	Electrocardiograph
e-CRF	Electronic case report form
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
EudraCT	European Clinical Trials Database
FBC	Full blood count
FDA	Food and drug administration
FiO_2	Inspired oxygen fraction
fMLF	formyl-methionyl-leucyl phenylalanine
FOXP3	Forkhead box P3
FRH	Freeman Hospital
g	Gram

GCP	Good Clinical Practice
G-CSF	Granulocyte-colony stimulating factor
GI	Gastrointestinal
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Good Manufacturing Practice
HAI	Hospital-acquired infection
HBSS	Hanks balanced salt solution
HDU	High dependency unit
HELICS	Hospitals in Europe Link for Infection Control through Surveillance
HIV	Human immunodeficiency virus
HLA-DR	Human leukocyte antigen – D related
HR	Heart rate
ICAM	Intracellular adhesion molecule
ICNARC	Intensive Care National Audit and Research Centre
ICU	Intensive care unit
ICUAI	Intensive care unit-acquired infection
IFN-g	Interferon gamma
IL	Interleukin
IL-1β	Interleukin 1 beta
IMDM	Iscove's modified dulbecco medium
IMP	Investigational Medicinal Product
IQR	Interquartile range
ISF	Investigator site file
ISRCTN	International standard randomised controlled trial number
ITAM	Immunoreceptor tyrosine-based activation motif

ITT	Intention to treat
kg	Kilogram
kPa	Kilopascal
L	Litre(s)
LDN/G	Low density neutrophil / granulocyte
LFTs	Liver function tests
LPS	Lipopolysaccharide
µg	Microgram
MAC-1	Macrophage-1 antigen
MAP	Mitogen-activated protein
MAP	Mean arterial pressure
mg	Milligram
ml	Millilitres
mmHg	Millimetres of mercury
MHC	Major histocompatibility complex
µg	Microgram
µmol	Micromole
MHRA	Medicines and Healthcare Products Regulatory Agency
MV	Mechanical ventilation
N/A	Not applicable
NADPH	Nicotinamide adenine dinucleotide phosphate
NCTU	Newcastle Clinical Trials Unit
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHS	National health service
NIHR	National Institute for Health Research
NIV	Non-invasive ventilation

OSA	Obstructive sleep apnoea
PAF	Platelet activating factor
PaO_2	Partial pressure of oxygen in arterial blood
PAMPS	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PE	Phycoerythrin
PerLR	Personal legal representative
PI	Principal investigator
PI	Propidium iodide
PI3K	Phosphatidylinositol-3-kinase
PMG	Project management group
ProfLR	Professional legal representative
QEH	Queen Elizabeth Hospital
R&D	Research and Development
RCT	Randomised controlled trial
REC	Research Ethics Committee
rhu GM-CSF	Recombinant human granulocyte-macrophage colony-stimulating factor
ROS	Reactive oxygen species
RVI	Royal Victoria Infirmary
SAE	Serious adverse event
SaO_2	Oxygen saturation in arterial blood
SAR	Serious adverse reaction
SCCM	Society of Critical Care Medicine
S/C	Subcutaneous
s.d.	Standard deviation

SDF-1	Stromal derived factor-1
SIRS	Systemic inflammatory response syndrome
SLE	Systemic lupus erythematosus
SMF	Study master file
SOFA	Sequential organ failure assessment
SOD	Superoxide dismutase
SOP	Standard operating procedure
SmPC	Summary of product characteristics
SUSAR	Suspected unexpected serious adverse reaction
TGF	Transforming growth factor
TLR	Toll like receptor
TNF	Tumour necrosis factor
T reg	Regulatory T cells
U	Unit
U&Es	Urea and Electrolytes
U.K.	United Kingdom
US	United States
WCC	White cell count

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Chapter One: Introduction

1.1 Thesis Outline

Nosocomial infection is a major healthcare problem, particularly on the intensive care unit (ICU) where it can affect 20-40% of all admissions. Nosocomial infection has been shown to be associated with increased morbidity, mortality and health care costs. Despite multiple different strategies being introduced to tackle this problem nosocomial infection rates remain high.

There is increasing evidence for the development of immunosuppression during critical illness and studies have shown that the development of such acquired defects in the innate immune system is associated with increased risk of developing nosocomial infection.

This study focuses on neutrophil impairment during critical illness, and aimed to investigate whether impaired neutrophil phagocytosis may be restored with administration of, the immunostimulatory drug, granulocyte-macrophage colony stimulating factor (GM-CSF). If GM-CSF were able to restore neutrophil function it may have a role in preventing nosocomial infection in the ICU.

Chapter 1, the introduction to the thesis, will outline the background to the study reviewing: the evidence for the the impact of nosocomial infection, the pathogenesis of sepsis and the systemic inflammatory response; the pathophysiology of immune dysfunction in sepsis and critical illness in relation to neutrophil, monocyte and T lymphocyte dysfunction in particular; the recognition and development in the understanding of GM-CSF and its therapeutic effects in treating illness and disease.

Chapter 2 of the thesis will outline the materials and methods used in developing and completing the study including the necessary regulatory approvals, the design of each phase of the study and the clinical and laboratory procedures undertaken.

Chapter 3 will describe the results of the preliminary phase of the study, an assay confirmation study, designed to locally validate the use of a surrogate biomarker for neutrophil phagocytic dysfunction. It will discuss the results and how they impacted on the design of the subsequent dose finding study and randomised controlled trial.

Chapter 4 will outline the results of the dose finding study, undertaken to ensure a

safe and effective dose would be carried through to the randomised controlled trial. Finally, chapter 5 will outline the results and conclusions of the randomised controlled trial and suggest future areas of study.

1.2 Background

1.2.1 Nosocomial infection

Hospital-acquired infection (HAI), also known as nosocomial infection, affects up to 10% of hospital inpatients at any one time (Vincent et al., 1995; Vincent et al., 2009; Health Protection Agency., 2001; Pratt et al 2001). Nosocomial infections are defined as those which are not present or incubating prior to admission to a healthcare setting. Infections occurring after the first 48 hours of admission are generally considered to be nosocomial (World Health Organization, 2002). Rates of nosocomial infection among critically ill patients on the intensive care unit (ICU) are considerably higher than in the rest of the hospital population with 3-5 times as many patients (20 - 40% of all ICU admissions) developing at least one secondary infection during their stay (Health Protection Agency., 2001; Sanchez-Velazquez et al., 2006; Donowitz et al., 1982).

The development of such infections places a significant burden on both individual patients and the health care system as a whole with increased morbidity, mortality and health care costs having been shown to be directly attributable (Ylipalosaari et l.,2006; Sanchez-Velazquez et al., 2006; Olachea et al.,1995; Digiovine et al., 1999). Nosocomial infection accounts for approximately 5000 deaths annually within the UK (Inweregbu et al., 2005) and estimates of total annual cost to the NHS of care related to such infections are in the region of £1 billion (Health Protection Agency., 2011). Large scale studies have shown that up to 1/3 of nosocomial infections are avoidable (Haley et al., 1985) and strategies for preventing hospital-acquired infections have been the focus of government health policy makers in recent years. Within the UK each hospital Trust is required to have a dedicated infection control team responsible for ensuring compliance with infection control guidelines and meeting set targets in relation to HAI (Department of Health, 2008, 2015).

1.2.2 Nosocomial infection within the intensive care unit

There are several reasons why critically ill patients in intensive care are particularly vulnerable to nosocomial infection. The EPIC study, published in 1995, was a large scale Europe-wide point prevalence study involving more than 4,500 patients on intensive care. It set out to establish not only the prevalence of ICU- acquired infections, but also the risk factors contributing to the development of such infections and the associated consequences. Several factors were found to increase the risk of nosocomial infection and could be broadly divided into 4 distinct groups; underlying health status, the acute disease process, invasive procedures and treatments (Vincent et al.,1995). Patients on ICU have greater levels of underlying chronic diseases, for example diabetes, chronic lung disease and ischaemic heart disease, many of which contribute to relative immunosuppression (Sreeramoju et al., 2008, Lola et al., 2011). The nature of the acute disease process resulting in admission to ICU can also affect the risk of developing a nosocomial infection with patients presenting with sepsis, burns, trauma or those requiring surgery having increased risk (Vincent et al., 1995, Sreeramoju et al., 2008). Invasive procedures breach the body's natural protective mechanisms and indwelling catheters at any site provide passageways and reservoirs for infection. The EPIC study revealed increased rates of infection in the presence of endotracheal tubes, central venous catheters, nasogastric tubes and urinary catheters (Vincent et al., 1995). 64% of all blood stream infections recorded in UK point prevalence data were associated with some form of vascular access device in the previous 48 hours.(Health Protection Agency., 2011) Finally, many of the treatments administered to ICU patients are associated with increased rates of nosocomial infection including blood transfusion (Hill et al., 2003), immunosuppressive drugs, stress ulcer prophylaxis and in particular repeated antibiotics. A significant proportion of such patients will have received multiple antibiotics during their hospital stay, leading to the development of resistant organisms and making such infections more difficult to treat. One study found that 70% of ICU-acquired infections (ICUAI) were due to organisms resistant to one or more antibiotic,(Burke et al., 2003) with another retrospective analysis of eight years of Gram negative blood stream infections showing 30-50% resistance to one or more antibiotic (Sligl et al., 2015).

1.2.3 Prevention of Nosocomial Infection

Large scale surveillance programmes including the Hospitals in Europe Link for Infection Control through Surveillance (HELICS) programme and the UK Nosocomial Infection National Surveillance Service have been established to monitor the incidence of HAI and identify intervention targets for reducing rates and improving patient care. The establishment of infection control teams, development of care bundles and improvements in hand hygiene and sterile technique have delivered some success and as a consequence rates of nosocomial infection have fallen over the last 10 years, with prevalence falling from 9.2% in 1980 to 6.4% in 2011. (Health Protection Agency., 2011 (see Table 1); Pratt et al., 2001)

Prevalence study	Total number of patients	No of patients with HAI	Prevalence %	95% confidence interval
1980 UK	18163	1671	9.2	8.8 - 9.6
1993/4 UK	37111	3353	9.0	8.8 – 9.3
2006 England	58775	4812	8.2	8.0 – 8.4
2011 England	52443	3360	6.4	4.7 – 8.7

Table 1. Hospital-acquired infection prevalence surveys in England and UK (Adapted from Health Protection Agency English National Point Prevalence Survey on Healthcare-associated Infections and Anti-Microbial Use 2011).

Despite this, however, there is still an unacceptably high rate of nosocomial infections occurring nationally and worldwide and other possible mechanisms need to be explored in an attempt to eliminate this problem.

1.2.4 Immune dysfunction and nosocomial infection

Bacterial pathogens are responsible for the majority of HAIs, although, over the last 20 years fungi have been identified as contributing to an increasing proportion of infections (Monneret et al., 2011; Martin et al., 2003). Within a healthy functioning immune system, the neutrophil is one of the key cells in ensuring the rapid detection and clearance of pathogenic bacteria and fungi. Over recent years increasing interest has been generated in the area of immune dysfunction in sepsis and critical illness with strengthening evidence for an association between such acquired abnormalities of immune function and an increased risk of nosocomial infection (Monneret et al., 2011; Angele et al., 2002; Conway Morris et al.,2013). Several studies have demonstrated significantly increased rates of nosocomial infection in patients with evidence of immunoparesis raising the question of whether stimulation of the immune system may have a role in the prevention of nosocomial infection and potential strategies for addressing this have been explored (Monneret et al., 2011; Conway Morris et al., 2011, Meisel et al., 2009). Currently however there remains insufficient evidence to recommend any of these immune modulating treatments and a recent Cochrane meta-analysis suggested further trials were necessary (Bo et al., 2011).

The role of immune dysfunction in critical illness and in particular acquired impairment of neutrophil phagocytosis, will be the focus of this work.

1.3 Critical illness, sepsis and the systemic inflammatory response

Critical illness, generally accepted to mean a life-threatening illness, is usually accompanied by a marked systemic inflammatory response syndrome (SIRS). Sepsis (SIRS with infection), severe sepsis and septic shock form a continuum of a hyper-inflammatory state resulting in hypoperfusion, impairment in oxygen delivery and ultimately organ dysfunction and failure. The understanding of the pathophysiology of the systemic inflammatory response and sepsis has evolved over many years.

In 1991 The American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM) held a consensus conference with the purpose of producing a framework which would help to define sepsis and the systemic inflammatory response. (Members of the ACCP/SCCM Consensus Conference

Committee, 1992) They drew up a set of criteria with which to define the systemic inflammatory response and the stages of sepsis. The guidance published in 1992 was widely accepted and has been used throughout the world to aid clinical decision making and as a tool to define eligibility for research studies.

The systemic inflammatory response is considered to be present when two or more of the following parameters are clinically evident:

i. temperature greater than 38°C or less than 36°C
ii. white cell count less than 4 or greater than $12x10^9$/L
iii. heart rate greater than 90 beats per minute
iv. respiratory rate greater than 20 breaths per minute or $PaCO_2$ less than 32mmHg

It is recognised that the systemic inflammatory response is not specific for sepsis but also occurs in the context of non-septic inflammation such as acute pancreatitis, trauma and thermal injury.

A second consensus conference, The International Sepsis Definitions Conference, was held in 2001 as there were concerns regarding the lack of specificity of the SIRS criteria. After 10 years of further research into sepsis, with the discovery of novel biomarkers and increased understanding of the multiple mediators implicated in the pathophysiology of sepsis, it was felt that the definitions should be updated to include such advances in knowledge. Following extensive review of the literature and subsequent expert debate it was concluded that despite extensive research no definitive evidence existed to allow such parameters to be incorporated into guidelines, however, additional clinical features were added to more clearly define the real clinical situations encountered (Levy et al., 2003).

As will be discussed, the systemic inflammatory response arising in sepsis and critical illness is responsible for the acquired immune dysfunction seen.

1.3.1 Epidemiology of sepsis

Sepsis causes significant health problems world wide and is a leading cause of death in critically ill patients (Mayr et al.,2014). With an increasingly ageing population affected by co-morbid disease and impaired immunity as a result of immunosenescence (progressive deterioration of the immune system with ageing (Berrut G et al., 2015)) the incidence of sepsis has been increasing over the last few decades. A secondary analysis of data gathered between 1996 and 2004 on the Intensive Care National Audit and Research Centre (ICNARC) Case Mix Programme Database revealed that of the 343,860 admissions to 172 ICUs in England, Wales and Northern Ireland 92,672 (27%) met the criteria for severe sepsis within the first 24 hours of admission. The number and percentage of admissions with severe sepsis rose over the course of the study from 46 per 100,000 of the adult population (23.1% of admissions), in 1996 to 66 per 100,000 of the adult population (28.7% of admissions), in 2004 (Harrison et al., 2006). Although there was a reduction in the mortality rate among patients with severe sepsis, from 48.3% in 1996 to 44.7% in 2004, as a result of improvements in care, overall number of deaths increased due to the increased patient numbers (Harrison et al., 2006). With advances in treatment protocols, increasing numbers of patients survive the early pro-inflammatory phase with the majority of deaths occurring late in the course of sepsis as a result of acquired immunosuppression leading to secondary nosocomial infection (Otto et al., 2011).

1.3.2 Pathophysiology of sepsis and the systemic inflammatory response

Traditionally sepsis was believed to be characterised by overwhelming pro-inflammatory cytokine release, often termed a 'cytokine storm' following recognition of foreign pathogens and activation of the innate immune system. It was understood that this resulted in recognised clinical signs and symptoms such as fever, tachycardia and hypotension. This theory was supported by studies in animals using large doses of lipopolysaccharide (LPS) or bacteria where very high levels of circulating cytokines were recorded and death occurred quickly as a result of an overwhelming response. In these studies, improvements in mortality could be seen when such inflammatory mediators were blocked calming the cytokine storm. (Riedemann et al., 2003; Deitch, 1998)

Multiple pro-inflammatory mediators were subsequently identified as components of the systemic inflammatory response (e.g.tumour necrosis factor alpha (TNFα), interleukin 1 beta, (IL-1β), interleukin 6 (IL-6),interleukin 8 (IL-8), inteferon gamma IFN-g) and became the targets of research into sepsis prevention and control. Many trials set out to investigate the role of anti-inflammatory agents in combating sepsis (see table 2) but while targeting specific pro-inflammatory mediators had been shown to be of benefit in animal models, trials in humans failed, with some resulting in worse outcomes. (Zeni et al., 1997; Natanson et al., 1994;). It became apparent that targeting the blockade of a single inflammatory agent would not be effective in controlling the host response.

Target Year (ref)	Intervention	Population	Primary Outcome	Result
IL-1 1994[a]	Recombinant human IL-1 receptor antagonist	893 patients sepsis syndrome	28 day all cause mortality	No significant difference in survival (? Increased survival if >1 organ dysfunction – secondary analysis)
IL-1 1997[b]	IL-1 receptor antagonist	696 patients severe sepsis / septic shock	28 day all cause mortality	No significant difference
TNF – α 2001[c]	Lenercept – TNF – α receptor fusion protein	1342 patients 73% severe sepsis 27% septic shock	28 day all cause mortality	No significant difference
TNF – α 2004[d]	Monocloncal TNF – α antibody	2634 patients severe sepsis (998 ↑ IL-6 levels)	28 day all cause mortality	Overall no difference in mortality, mortality reduced in sub-group with increased IL-6
TNF – α 2006[e]	Affinity purified anti TNF – α fAb	81 patients septic shock /2 organ dysfunction	Reduction in TNF – α and IL-6 levels	↓ plasma TNF- α and IL-6. No difference in shock free days, 28 day mortality 37% placebo vs 26% FAb
Multiple 1987[f]	High dose methylprednisolone	382 patients with sepsis	Reduction in shock / mortality	No significant difference
TLR4 2013[g]	Eritoran MD2-TLR4 Lipid A antagonist	1961 patients with severe sepsis	28 day all cause mortality	No significant difference in survival

Table 2: Trials targeting pro-inflammatory mediators of sepsis. [a]Fisher et al., 1994; [b]Opal et al., 1997; [c]Abraham et al., 2001; [d]Panacek et al., 2004; [e]Rice et al., 2006; [f]Bone et al; [g]Opal et al 2013.

1.3.3 Immune dysfunction in sepsis

As early as the 1970s it was noted that in addition to the inflammatory response patients with sepsis and trauma frequently displayed features of immune impairment, initially demonstrated by a loss of delayed type hypersensitivity response to common antigens (Meakins et al.,1977). Further studies showed that whole blood and isolated immune cells from septic patients had a lower capacity for producing inflammatory mediators (Hotchkiss et al., 2013). It was also seen that mortality rates were worse in patients where anti-inflammatory cytokines predominated (Van Dissel et al., 1998). Gradually there was increased recognition of the importance of the fact that that the exaggerated inflammatory response was very often accompanied by an anti-inflammatory response with certain patients who displayed marked anti-inflammatory response having poor outcomes (Boomer et al., 2011; Schefold et al., 2008). Similar patterns were also recognised in patients with other non-infective forms of critical illness including burns, trauma, pancreatitis and massive transfusion (Schwacha et al., 2002; Hazeldine et al.,2014; Angele et al., 2002; Cata et al., 2013, Hill et al., 2003). This became known as the compensatory anti-inflammatory response.

1.3.4 Compensatory anti-inflammatory response syndrome

Bone first described the phenomenon of the Compensatory Anti-inflammatory Response Syndrome (CARS), in the mid 1990s and went on to characterize it in more detail (Bone,1996; Bone et al., 1997). He noted that the onset of the anti-inflammatory response appeared early in the course of sepsis but that it developed more slowly, than the pro-inflammatory response, and appeared to persist for longer (Figure 1). He hypothesized that the role of the anti-inflammatory response was to control and regulate the inflammatory response with the aim of restoring homeostasis. Anti-inflammatory mediators associated with this response were identified in increased concentrations including IL-10, IL-4, IL-13, TNF receptor and IL-1β receptor antagonist (Gogos et al., 2000). The effects of these anti-inflammatory mediators include down regulation of monocyte MHC class II expression, a reduction in antigen presentation and a decrease in cytokine release from various cells of the innate immune system (Bone et al., 1997). Plasma from such patients has been shown to act as an immunosuppressive medium when applied to healthy cells

(Cavaillon et al., 2001). Bone proposed that the reason for the failure of clinical trials targeting specific pro-inflammatory mediators was related to imbalances in the pro and anti-inflammatory response and that beyond the very acute phase of illness it was often the effects of the anti-inflammatory response which led to secondary infection and death (Bone et al.,1997(a); Bone , 1997(b)).

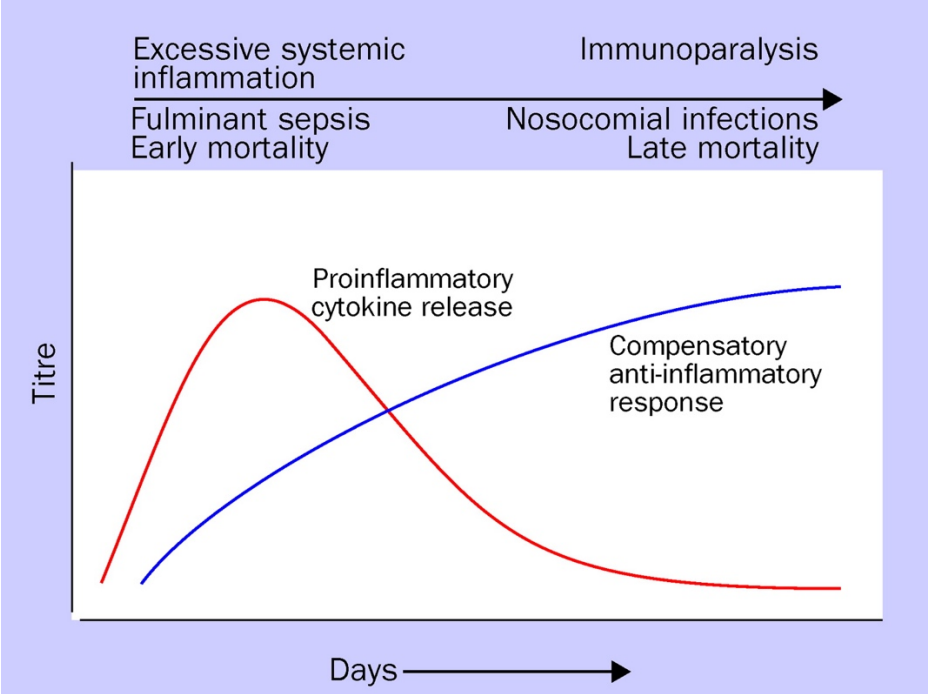

Figure 1a: Compensatory anti-inflammatory response theory in sepsis. Reprinted from The Lancet Infectious diseases. Vol. 1, Van der Poll. Immunotherapy of sepsis, Pages 165-174. Copyright (2001), with permission from Elsevier.

1.3.5 Current understanding

It became clear that sepsis and the systemic inflammatory response was a vastly complex event with the course of the disease depending upon the balance between the pro and anti-inflammatory response (Reinhart et al., 2012). Current understanding is that microbial pathogen associated molecular patterns (PAMPS) activate cells of the innate immune system though pattern recognition receptors (PRRs) stimulating an acute phase response. In the case of non-septic inflammation, the process is triggered by danger associated molecular patterns

(DAMPS) released during tissue damage and cellular necrosis (Boomer et al., 2014). Several families of PRRs have been identified including Toll-like receptors (TLR), RIG-I-like receptors, NOD-like receptors and DNA receptors (Kumar et al., 2011). Binding with the pattern recognition receptors such as the Toll Like Receptor (TLR) family causes activation of immune cells and in the case of TLR 4 triggers nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and the mitogen-activated protein (MAP) kinase pathway resulting in the release of multiple inflammatory mediators including cytokines, chemokines, components of the complement system and vasoactive proteins (Stearns-Kurosawa et al., 2011). NOD-like receptors form inflammasomes which have a significant role in the secretion of IL-1ß and IL-18 (Gotts et al., 2016). Cytokines cause an increase in adhesion molecules on endothelial surfaces recruiting additional cells to the site of inflammation strengthening the cascade. At the same time enhanced phagocytosis of opsonized bacterial pathogens by neutrophils and macrophages takes place producing further inflammatory cytokine release. (Stearns-Kurosawa et al., 2011, Kumar et al., 2011).

In addition to stimulating the release of pro-inflammatory mediators, activation of cells in the innate and adaptive immune system leads to release of anti-inflammatory mediators driving the immunosuppression seen. Both macrophages and neutrophils release IL-10 and TGF-ß which have anti-inflammatory effects (Opal et al., 2000). During sepsis, interactions of antigen presenting cells with cells of the adaptive immune system contribute to the anti-inflammatory effects with a shift in the balance of T cells from Th1 cells releasing pro-inflammatory cytokines such as TNF- α, IL-2, IL-12 and IFN-g to Th2 cells releasing the anti-inflammatory mediators IL-4, IL-5, IL-9 and IL-10. (Aziz et al. 2013, Boomer et al., 2014).

The combined effects of these anti-inflammatory mediators constitutes the compensatory anti-inflammatory response. Studies of the effects of this anti-inflammatory response have revealed defects in all cells of the innate immune system.

1.4 Role of neutrophils in host defense

Neutrophils are the key cells responsible for the clearance of bacterial and fungal infection. Within their role in the innate immune system they act through various mechanisms to engulf and destroy pathogens. Disease states in which abnormalities in either number or function of neutrophils occur confirm the importance of neutrophils in protection against serious infection. Congenital neutropaenia encompasses a range of diagnoses of varying severity all of which show increased tendency to serious infection and prior to the era of G-CSF often resulted in death in early infancy (Donadieu et al., 2011). Chronic granulomatous disease, a condition in which neutrophils display impairment in the respiratory burst and production of oxygen free radicals due to mutations in the genes responsible for Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, is characterised by recurrent severe bacterial and fungal infections (Heyworth et al., 2003). Similarly individuals with leukocyte adhesion deficiency disorders, where a defect in β2 integrins prevents firm adhesion to and migration across the endothelial wall of blood vessels and subsequent failure of phagocytosis, display increased susceptibility to infection (Anderson et al., 1985).

1.4.1 Neutrophil production and release

Neutrophils are produced and released from the bone marrow at a rate of up to $2x10^{11}$ per day (Kolaczkowska et Kubes.,2013). Their production is under control of the cytokine granulocyte-colony stimulating factor (G-CSF). Mice lacking G-CSF have been shown to be neutropaenic (Lieschke et al., 1994), and G-CSF therapy is currently utilized in congenital neutropaenia and acquired neutropaenia, e.g. post chemotherapy, to restore circulating neutrophil numbers (Donini, 2007; Mehta et al., 2015; Metcalf, 2010).

The subsequent release of neutrophils from the bone marrow is regulated by CXCR4 and SDF-1(stromal derived factor – 1) which maintain homeostasis by retaining the cells in the bone marrow until required (Summers et al., 2010). Once in the systemic circulation it is thought that neutrophils have a relatively short half-life moving to the tissues or undergoing apoptosis within hours, however, more recent research has suggested that they may persist for up to 5 days (Pillay et al., 2010), even under basal conditions, although the experimental methods used to

measure neutrophil survival in these data have been debated. What seems apparent, however, is that when neutrophils are activated their survival is increased enabling primed neutrophils to remain present at sites of inflammatory insult for longer (Witko-Sarsat et al., 2011).

Following release of neutrophils into the circulation approximately 50% of the cells have been noted to 'disappear' and have been shown to be transiting slowly through large organs forming the intravascular 'marginated pool' residing within the liver, spleen, bone marrow and lungs (Summers et al., 2010). These cells can be recruited into the circulation at any time in response to pathogen or tissue-derived molecular signals.

During the inflammatory response neutrophil number increases markedly due to the secretion of G-CSF which interferes with the CXCR4-SDF-1 retention signal resulting in release of increased numbers of neutrophils from the bone-marrow (Wengner et al., 2008). Upon release from the bone marrow neutrophils rapidly migrate from the circulation into affected tissues under the influence of chemotactic cytokines released by macrophages and dendritic cells that reside in the tissues and trigger the neutrophil response.

1.4.2 Neutrophil recruitment, chemotaxis and extravasation

Multiple mediators are involved in coordinating the passage of neutrophils from the circulation to the site of inflammation and injury. Circulating neutrophils are attracted to the endothelial cell surface by cell adhesion molecules called selectins (type 1membrane glycoproteins which have a characteristic NH2 – terminal lectin domain and an EGF-like domain), a process known as tethering. Pattern recognition receptors on the endothelial surface detect the presence of pathogens and up regulate the expression of both platelet α (P)-selectin and endothelium (E)-selectin which bind to their glycosylated ligands on the neutrophil, in particular PSGL1 maximising neutrophil capture and recruitment (Kolaczkowska et Kubes.,2013). Once tethered the neutrophils initiate a 'rolling' mechanism whereby the cells are loosely adhered to the vessel wall and transition along it in response to chemotactic factors Figure 1b). Breakage and reformation of the selectin-ligand bonds allow smooth rolling of the neutrophil despite the shear forces of the passing blood flow (Ramachnadran et al., 2004). During the rolling process, as a result of exposure to

stronger, more specific chemotactic signals, in particular CXCL8 (IL-8), the neutrophils can become primed and activated. Via high affinity binding between β2 integrins and glycoproteins they can then firmly adhere to the endothelial cell surface. β2 integrins, the cell surface molecules responsible for tight adhesion to the endothelium, are composed of variable α subunits CD11a/CD11b/CD11c and a common β subunit, CD18. CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac1) are 2 important β2 integrins on the neutrophil surface and bind via intracellular adhesion molecule (ICAM) ligands mediated by calcium- and magnesium-dependent pathways resulting in tight adhesion (Burg et Pillinger.,2001). Murine studies examining deficiency of either integrin show marked impairment of adhesion (Burg et Pillinger.,2001) and in humans, impairment of LFA-1 and Mac-1 results in adhesion disorder and functional impairment of neutrophils (Anderson et al., 1985).

Once the neutrophil has adhered to the endothelium it begins the process of transmigration across the endothelial lining into the affected tissues (Figure 2). Transmigration can occur at the endothelial cell-cell junctions or transcellularly, the former being the preferred, more efficient route (Kolaczkowska et Kubes.,2013; Burg et Pillinger.,2001). Neutrophils crawl along the endothelial surface in a manner which is both chemokine dependent and independent. Neutrophils are also seen to crawl in a direction perpendicular to the flow of blood in a non-chemokine-dependent nature directly towards the site of transmigration. Transmigration is dependent on integrins, in particular integrin associated protein (IAP) and platelet/endothelial cell adhesion molecules and passage takes about 10-20 minutes. This happens across a chemotactic gradient (Kolaczkowska et Kubes.,2013; Burg et Pillinger., 2001; Hellewell, 2003).

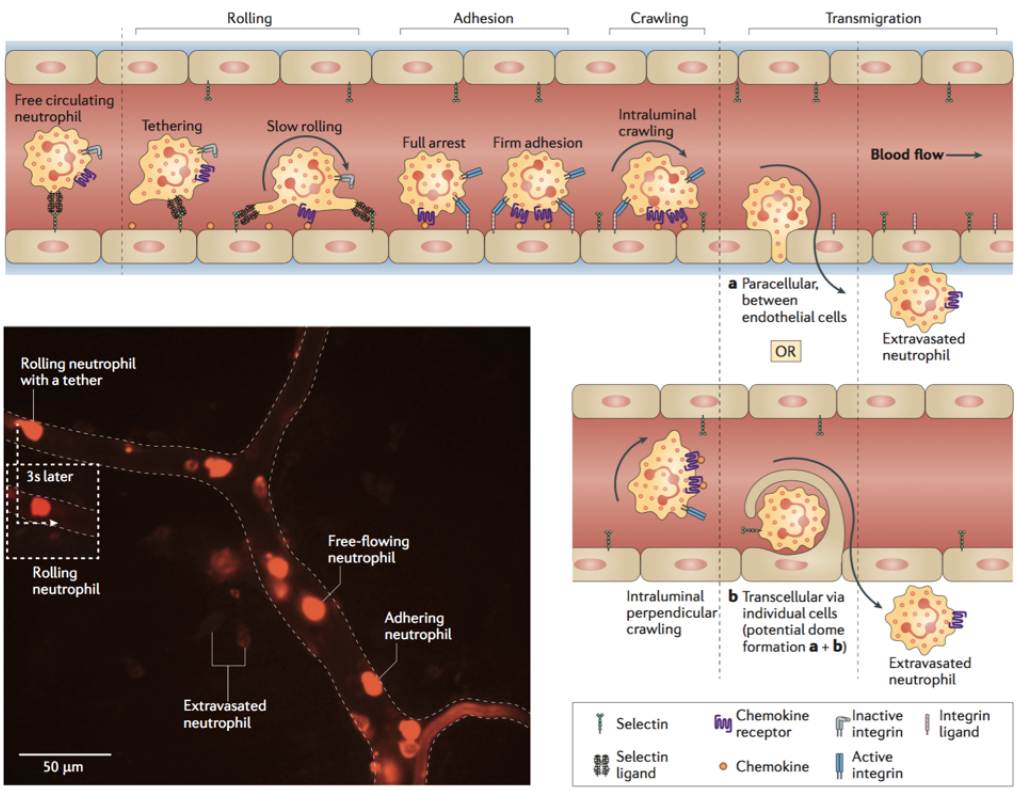

Fig 1b: The updated classical neutrophil cascade. Shown are the sequential steps of neutrophil recruitment from the vasculature to the tissue. Two possible methods of transmigration are acknowledged: paracellular (between endothelial cells; **a**) and transcellular (through endothelial cells; **b**). Major groups of adhesion molecules are marked. Rolling is mostly selectin-dependent, whereas adhesion, crawling and transmigration depend on integrin interactions. Chemokines lining the luminal part of endothelium activate rolling neutrophils, thus inducing conformational changes of neutrophil surface integrins and allowing for subsequent events. Crawling neutrophils follow the chemokine gradient along endothelium, which guides them to the preferential sites of transmigration. The intravital microscopy image shows a skin postcapillary venule with neutrophils ($LY6G^+$ cells) labelled in red (phycoerythrin antibody conjugate; 10 µg). Mouse skin was infected with *Staphylococcus aureus* and the image was taken 2 hours later. It captured neutrophils at different stages of migration: freely circulating cells, rolling cells extending tethers, adhering neutrophils and the cells that extravasated out of the blood vessel. Reprinted by permission from Macmillan Publishers Ltd : Nat Rev Immunol. 2013;13(3):159-75. Copyright (2013).

1.4.3 Bacterial killing; Phagocytosis and superoxide release

Once within the target tissue the neutrophil begins the process of phagocytosis and pathogen destruction, following a new chemotactic gradient, created by molecules such as formyl-methionyl-leucyl phenylalanine (fMLF) or C5a, leading to the 'end-target' infecting particle. Neutrophils can phagocytose both opsonised and non-opsonised particles. Opsonised phagocytosis can be both complement and immunoglobulin mediated. The Fc receptors CD32 and CD16 present on resting neutrophils bind to immunoglobulin while a subset of β2 integrins (MAC1) binds complement coated particles (Lee et al., 2003). In order for the activation of Fc receptors to take place phosphorylation of tyrosine residues within an immunoreceptor tyrosine activation motif (ITAM) must take place (Lee et al., 2003). This process relies on activation of Src-family kinases (Kiefer et al., 1998). Following phosphorylation, binding of tyrosine kinase Syk on generated sites stimulates phosphatidylinositol-3-kinase (PI3K) which in turn converts phosphatidylinositol 4,5-biphosphate to phosphatidylinositol 3,4,5-triphosphate. This cascade leads to the polymerization of actin and activation of myosin necessary for ingestion of the foreign particle (Dale et al., 2008). Once bound, the foreign particle is ingested with or without the extension of pseudopods to form a vacuole known as a phagosome. Once inside the cell the phagosome fuses with lysosomes (specific granules containing cytotoxic enzymes) induced by a rise in intracellular calcium levels and activation of protein kinases (Dale et al., 2008). Neutrophil granules are of key importance in the processes of bacterial killing and digestion of the pathogen which follows. The processes are also dependent on the oxidase NADPH, contained in secondary granules which is essential to the processes of microbial killing (Segal, 2005; Lee et al., 2003). Via the transfer of negative charge from NADPH contained in the cytosol to oxygen molecules within the phagosome superoxide anions are created in large numbers (Lee et al, 2003). This marked increase in oxygen consumption with the production of oxygen free radicals is known as the respiratory burst. The oxygen metabolites, so called reactive oxygen species (ROS), produced are toxic to microbes and result in effective killing. Secretion of biologically active cytotoxic substances released from neutrophil granules either into the phagosome or into the extracellular milieu also contribute to the killing powers of neutrophils (Segal., 2005). During maturation 3 different types of granules are produced all of which play

an important role in the neutrophils response to pathogens. The primary, or azurophilic, granules contain MPO, Cathepsin G, elastase and proteinase 3 all of which can effect pathogen digestion. MPO in particular is important in the conversion of superoxide to the more toxic hypochlorous acid which has increased killing anti-microbial effect (Borregaard et al.,1997). The secondary, or specific granules contain lactoferrin, transcoblamin and gelatinase and are more important in terms of restoring membrane components and preventing unwanted free radical reactions. The secondary and tertiary granules contain the flavocytochorme b558 which is a vital component of NADPH oxidase (Borregaard et al., 1997). In addition, activated neutrophils can exhibit extracellular killing with the release of NETs (neutrophil extra-cellular traps), containing core DNA and antimicrobial histones, proteins and enzymes. The NETs capture the pathogens and facilitate killing either via cytotoxic proteins and histones or by enabling phagocytosis to occur (Kolaczkowska et Kubes.,2013).

1.4.4 Neutrophil apoptosis

When the life span of the neutrophil has been reached it undergoes a process of programmed cell death known as apoptosis. Neutrophils undergoing apoptosis can be identified by several characteristic changes in their microscopic appearance including prominent densely condensed nuclei and vacuolated cytoplasm (Savill et al., 1989). Changes in the surface expression of phosphatydylserine, due to alterations in plasma membrane phospholipids, reveals apoptotic neutrophils to phagocytic macrophages responsible for their clearance (Fadok et al., 2001). The engulfment of apoptotic neutrophils by tissue macrophages is associated with inhibition of the secretion of pro-inflammatory cytokines (Fadok et al., 1998). Controlled cellular death, happening in this way, prevents the spilling of cytotoxic substances from the dying neutrophil. This protective process minimises damage to the surrounding environment and is an essential part of tissue recovery. Constitutive apoptosis of circulating neutrophils takes place in the liver, spleen and bone marrow (Saverymuttu et al., 1985). Neutrophil apoptosis is mediated by multiple factors but critically by the family of caspases with caspase 8 and 9 being responsible for initiation of apoptosis (Luo et al., 2008; McCracken et al., 2014). Another family of proteins, the Bcl-2 family, includes both pro-apoptotic proteins (Bid and Bax) and pro-

survival proteins (Mcl-1 and A1). These proteins play a key role in mitochondrial integrity affecting the release of further pro-apoptotic factors in the cascade. Apoptosis is also under the influence of multiple extracellular mediators many of which are increased during infection and inflammation when they exert a pro-survival effect leading to a delay in apoptosis. The balance of pro-survival and pro-apoptosis proteins determines the rate at which apoptosis occurs (Luo et al., 2008; McCracken et al., 2014). Apoptotic neutrophils have been shown to exhibit impairment in various aspects of neutrophil function including chemotactic response to stimulus, reduction in release of reactive oxygen species and impairment in phagocytosis (Whyte et al., 1993). The prolonged lifespan of neutrophils secondary to delayed apoptosis in sepsis and critical illness and has been shown to be affected by the presence of pro-inflammatory cytokines and hypoxia (Michlewska et al., 2009; Hannah et al., 1995). Although extended neutrophil survival may be beneficial in terms of enhancing the host response to infection delayed apoptosis is implicated in the tissue damage seen in many inflammatory diseases (Elmore, 2007; Luo et al., 2008)). The aforementioned externalisation of phosphatydylserine during the process of apoptosis is advantageous for research purposes where apoptotic neutrophils can be identified by Annexin V binding for flow cytometry analysis (Akgul et al., 2001).

1.5 Immune cell dysfunction in critical illness

1.5.1 Neutrophil dysfunction in critical illness

During critical illness the overwhelming inflammatory response leads to neutrophil dysfunction with impairment of the ability of the neutrophil to facilitate clearance of pathogens. Studies have shown defects in all of the key processes namely phagocytosis, chemotaxis, ROS and bacterial killing (Wenisch et al., 2001, Lu et al., 2016). There are some conflicting reports describing enhanced phagocytosis and ROS production in some septic patients which may be explained by the severity of illness and balance of the pro-inflammatory and anti- inflammatory responses in the patient cohorts studied (Martins et al., 2003; Hazeldine et al., 2014). The release of large numbers of immature neutrophils into the circulation, due to the effect of inflammatory cytokines on CXCR4/CXCL12 signaling in the bone marrow probably

contributes to neutrophil impairment (Cummings et al. 1999). Immature neutrophils have been shown to display significantly reduced phagocytic capacity when compared to neutrophils from healthy volunteers (Taneja et al., 2007; Pillay et al., 2010). Heterogeneity in the functional capacity of neutrophils in sepsis and critical illness may be due to alterations in membrane receptors vital for these processes (Pillay et al., 2010; Tarlowe et al., 2005).

Conway Morris et al, previously confirmed that neutrophil phagocytosis is impaired in our target population of critically ill patients when compared with healthy volunteers. Studying 68 patients on intensive care, all of whom had suspected ventilator-associated pneumonia (VAP), a major ICU-acquired nosocomial infection, they showed a 36% absolute reduction in median phagocytic capacity in the critically ill patients compared with 21 age and sex-matched healthy controls. (Conway Morris et al., 2009). This problem was compounded by the fact that patients' neutrophils were less capable of generating bactericidal reactive oxygen species (ROS) and had reduced migratory capacity to chemotactic stimuli (Conway Morris et al., 2009).

In addition to demonstrating that neutrophil phagocytosis was impaired, Conway Morris et al went on to show that patients with proven phagocytic dysfunction had a more than 5-fold increased risk of developing an ICU-acquired infection during their admission (Conway Morris et al., 2011).

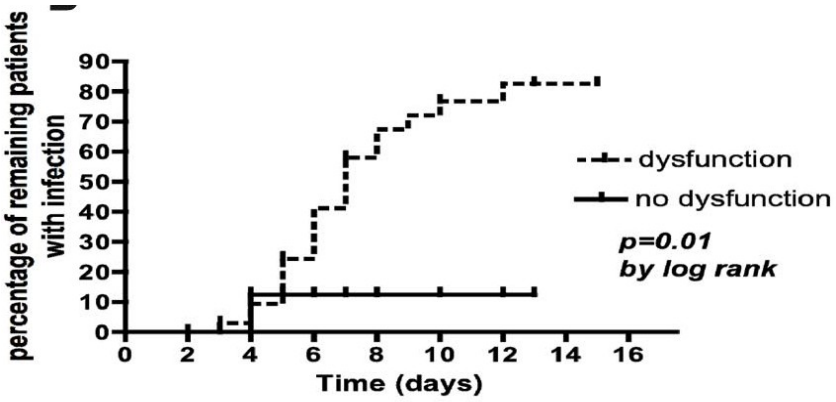

Figure 1c: Comparison of ICUAI in patients related to presence of neutrophil phagocytic dysfunction. Adapted from Conway Morris et al. Blood. 2011;117(19):5178-88.

Previous studies have also confirmed the association of phagocytic impairment with poor prognosis for patients with sepsis and septic shock (Danikas et al., 2008, Stephan et al., 2002).

1.5.2 Monocyte dysfunction in critical illness

Monocytes originate from progenitor cells in the bone marrow and are released into the circulation during septic and non-septic inflammation in response to various chemokines including CCL2 and CCL7.Accounting for approximately 10% of the circulating human leucocyte population they are involved in host antimicrobial defense including antigen presentation, removal of apoptotic cells and scavenging of toxins (Auffray et al., 2009). In addition, they have been implicated in the pathogenesis of many inflammatory conditions including atherosclerosis, rheumatoid arthritis and multiple sclerosis (Shi and Palmer., 2011). Monocyte recruitment into the tissues is triggered by local factors and chemokine release and follows the previously described pattern of leucocyte migration including rolling, adhesion and transmigration. Once in the tissues monocytes can differentiate into both tissue macrophages and dendritic cells (Shi and Palmer., 2011). Monocytes have been subdivided into 3 types depending on their expression of the cell surface markers CD14 and CD16 and are described as classical monocytes $CD14^{++}$ $CD16^{low}$, non-classical monocytes $CD14^{+}$ $CD16^{++}$ and intermediate monocytes$CD14^{++}$ $CD16^{+}$ (Ziegler-Heitbrock et al., 2010).

Monocyte dysfunction in sepsis has been recognised for some time. Alterations in monocyte function in sepsis are characterised by a reduction in HLA-DR surface expression and an accompanying reduction in antigen presentation. Reduced HLA-DR expression on monocytes has been shown to correlate with poor outcome in terms of mortality and secondary infection (Docke et al., 2005; Cheron et al., 2010).

HLA-DR is a major histocompatibility complex (MHC) class II cell surface receptor responsible for antigen presentation which can be readily measured by flow cytometry. It is encoded by the HLA complex on chromosome 6 (Cajander et al., 2013). Low levels of monocyte HLA-DR have been observed in the majority of patients with sepsis at onset but restoration to normal levels within 7 – 10 days has been observed in survivors (Tschaikowsky et al., 2006), with persisting low levels of monocyte HLA-DR in sepsis having been shown to predict mortality (Monneret G. et

al., 2006). Similar patterns of monocyte dysfunction and anergy have also been seen in patients presenting with non-septic systemic inflammation for example in trauma and burns, and those presenting with ruptured aneurysms. Low levels of HLA-DR expression have also being shown to be a predictor of poor outcome in these circumstances (Cheron et al., 2010, Venet et al., 2007, Haveman et al., 2006).

Consequently, monocyte HLA-DR expression has been identified as a surrogate marker for immune dysfunction in critical illness and measurement of monocyte HLA-DR has therefore been adopted for use in clinical trials to identify patients with immunoparesis (Monneret et al., 2006, Hotchkiss et al., 2014, Meisel et al., 2009). Monocyte HLA-DR is commonly measured by flow cytometry and an international protocol has been developed by Docke et al in an effort to standardise results (Docke et al., 2005). Commercially available kits such as the Quantibrite^{TM} kit (BD Biosciences) have been developed to facilitate its measurement. Monocyte HLA-DR expression levels > 20,000 antibodies per cell are widely accepted to be associated with immunocompetence while levels below 5,000 antibodies per cell are considered to be in keeping with immunoparalysis. To increase clinical use and reduce variation between laboratories other means of measuring HLA-DR are currently being explored including polymerase chain reaction (PCR) (Cajander et al., 2013).

While monocyte HLA-DR expression is now an accepted surrogate marker for immunosuppression (Faix, 2013) the effect of restoration of mHLA-DR levels on immune function has not been demonstrated.

1.5.3 T Lymphocyte dysfunction in critical illness

Lymphocyte apoptosis has been shown to be increased in sepsis and critical illness with altered differentiation of lymphocyte subsets. Increased apoptosis accompanied by a reduction in Th1 lymphocytes without an increase in the Th2 subset leads to anergy or a state of unresponsiveness to antigens.(Hotchkiss et al., 2003; Hotchkiss et al., 2003 (2), Kessel et al., 2009).

In particular, alterations in T regulatory cell (T reg) dominance play a significant role in immunosuppression in sepsis. T regulatory cells were first described in the 1970s when they were known as suppressor cells (Gershon et al., 1972). They play a crucial role in suppressing abnormal immune responses such as in autoimmune disease and following transplantation. They exert their immunosuppressive effects

through 4 different modes i) through the secretion of anti-inflammatory cytokines including IL-10 and TGF-ß ii) through cytolysis by releasing granzymes, iii) by causing mechanical disruption and iv) by modulating dendritic cell maturation or function, necessary for the activation of effector T cells (Vignali et al., 2008).

T regulatory cells make up about 5-10% of the normal circulating T cell population but their relative number is significantly increased in sepsis and critical illness (Venet et al., 2009, Tatura et al., 2012). Increased lymphocyte apoptosis leads to an overall increase in the proportion of T regulatory cells, despite an overall reduction in T reg numbers, probably due to T regs having a greater resistance to apoptosis (Venet et al., 2004, Christaki et al., 2010). T regs appear to recover in number quicker than other T cell populations and the proportion of T regs is seen to rise several days after the onset of critical illness (Venet et al., 2009, Tatura et al., 2012). There has been increasing importance placed on the significance of T regs in sepsis and critical illness in recent years. While there is some evidence for a protective effect of T regs in sepsis (Hein et al., 2010; Kulhorn et al., 2013) several studies have demonstrated the development of secondary sepsis and poorer outcomes in patients with persistently elevated T reg proportions (Conway Morris et al., 2013, Venet et al.,2008, Monneret et al., 2003). It is thought that T regs may contribute to the state of lymphocyte anergy seen. (Venet et al., 2009)

T regs express CD4 and CD25 surface receptors and have been identified by the presence of the transcriptional factor forkhead box P3 (FOXP3) that appears to be necessary for T reg development, maturation and function. Although FOXP3 is a very reliable marker of T regs in mice its role in identifying T regs in humans is less straightforward. In addition, identifying the cells through the use of FOXP3 requires nucleus staining which takes several hours and makes it not a useful test for rapid identification. The low presence of CD127 can instead be used. (Hein et al., 2010; Venet et al., 2009, Tatura et al., 2012) T regs can be further subdivided into subsets memory and naïve T regs (Booth et al., 2010).

1.5.4 Combined immune cell dysfunction

As discussed the deleterious effects of an overwhelming inflammatory response on immune cells are not limited to neutrophil function. Significantly, it has also been shown that in episodes of combined dysfunction the risk of nosocomial infection rises in a cumulative manner with up to 75% risk of nosocomial infection in patients with evidence of neutrophil, monocyte and regulatory T-lymphocyte dysfunction. (Conway Morris et al., 2013.)

1.6 Personalised medicine approach

While previous studies in sepsis and critical illness have failed in seeking to find a single target therapy to be used globally across the critically ill population it has been increasingly recognised that there is marked heterogeneity in the host response to sepsis. It has been observed that many trials of immunomodulatory drugs in sepsis have delivered the intervention at a set dose, over an arbitrary period of time, without clear understanding of the underlying immune function of the patient (Reinhart et al., 2012). Understanding this heterogeneity is vital in ensuring that treatments and interventions can be appropriately targeted towards individual patients depending on their phenotypic response.

A large study, published in 2007, examining blood samples from patients with community-acquired pneumonia showed that cytokine profiles in more than 1800 patients presenting to Emergency Departments varied significantly over the course of their disease. Measurements of IL-6, TNF and IL-10 taken daily for 1 week and then weekly until discharge revealed high levels of IL-6 and TNF at presentation which fell quickly but remained elevated for more than a week (Kellum et al., 2007). A high proportion of patients were also seen to have significantly elevated levels of IL-10 throughout. The highest risk of death was seen in patients with high levels of both pro-inflammatory IL-6 and anti-inflammatory IL-10. Interestingly unbalanced pro- and anti-inflammatory profiles were rarely seen and were not associated with poor outcome. Significant proportions of patients had normal levels of both IL-10 (64% survivors and 42% non-survivors) and TNF (61% survivors and 53% non-survivors) throughout their disease course illustrating that host cytokine response profiles vary greatly and are not necessarily associated with poor outcome. These data suggested that anticytokine therapies may be ineffective in

many and even harmful in some and in addition that the failure of some treatments may be due to inadequate duration of therapy with the host cytokine response persisting for more than a week (Kellum et al., 2007).

There have been increasing numbers of studies published which have demonstrated key genetic differences in patients with disparate responses to sepsis. The Genomic Association in Sepsis (GAINS) study carried out in the UK recruited almost 400 patients with sepsis secondary to community-acquired pneumonia. They carried out gene expression analysis to look for evidence of heterogeneity in individual sepsis leucocyte transcriptomes. They demonstrated 2 distinct sepsis response signatures with a predominant immunosuppressive type once again being associated with a worse prognosis. They were able to confirm previous work showing that the state of relative immunosuppression occurring in some individuals' response to sepsis begins soon after sepsis onset (Davenport et al., 2016).

This marked heterogeneity of cytokine response and lack of response to anti-cytokine treatments has led the search to focus on differing targets in sepsis including cellular function.

As the neutrophil is the key cell in defending against bacterial and fungal infection it has been chosen as the focus of this research. This research will explore the potential for reversing the functional impairment seen in neutrophils during critical illness, in particular phagocytosis. To exclude the possibility of treating patients with adequate neutrophil dysfunction and therefore unlikely to gain additional benefit from the therapy, we wish to recruit and therefore target our intervention towards, only patients with confirmed neutrophil dysfunction.

Interventions in sepsis are required to be delivered in a timely manner and as such much focus is currently placed on the identification of biomarkers which might be used as a rapid diagnostic or point of care test to stratify patients and personalize treatment. Isolating neutrophils from whole blood to perform functional assessments involves multistep laboratory procedures. We therefore sought to utilise a suitable potential biomarker which could be validated for use in rapidly identifying patients with neutrophil phagocytic impairment for recruitment into the study.

1.6.1 The role of complement in immune dysfunction during critical illness.

There has been much interest in the role of complement in immune dysfunction during critical illness. The complement cascade has been recognized as playing an important role in the body's natural defense system. When regulated normally it enhances both the innate and acquired immune systems through clearance of immune complexes, opsonisation of pathogens and direct lysis of invading organisms through the formation of the membrane attack complex. (Guo et al., 2004)

There are three pathways of complement activation, namely the classical pathway, the alternative pathway and the lectin binding pathway. The pathways are activated through different mechanisms but all converge at the level of C3 to produce several cleavage products. These products act in differing ways to trigger immune cell function. (Guo et al., 2004)

Following activation of the complement system C3 and C5 are converted to the anaphylatoxins C3a and C5a. C3a is chemotactic for mast cells and eosinophils and induces the release of histamine and vasoactive mediators. (Ward., 2004) C5a exerts a more significant effect on granulocytes, monocytes and macrophages producing a strong chemotactic response. In addition, C5a can enhance neutrophil oxidative burst, phagocytosis (Mollnes et al., 2002) and release of granule enzymes and is also effective in reducing neutrophil apoptosis (Perianayagam et al., 2002; Guo et al., 2006), enhancing expression of adhesion molecules and activating the coagulation pathway. When activation occurs in a regulated manner a localised inflammatory response produces a net protective effect with successful clearance of invading pathogens. (Guo et al., 2004) This important protective role of C5a has been demonstrated in animal studies showing that mice with complement defects are very susceptible to sepsis and mount an inadequate defensive response. (Fischer et al., 1997)

During critical illness (sepsis, trauma, burns) however, all three complement pathways may be activated simultaneously resulting in excessive release of activated products into the systemic circulation producing adverse consequences, in particular impairment of the host innate immune responses.

Studies in rats have revealed increased survival and a reduction in the degree of bacteraemia when animals undergoing caecal ligation and puncture (CLP) have

been treated with anti-C5a antibodies. The mechanisms for improved survival appeared to relate to reversal of C5a-mediated defects in the respiratory burst and C5a-mediated impairment in phagocytosis (Huber-Lang et al., 2002). A similar study involving CLP rat models showed a reduction in mortality, bacterial load and improvement in superoxide generation when C5a was blocked. (Czermak et al., 1999) An attenuated response has also been seen when *Escherichia coli* was used to induce septic shock and acute respiratory distress syndrome (ARDS) in monkeys treated with anti-C5a antibody. 100% of the animals treated with anti-C5a antibody survived while 75% of those treated with control antibody died. (Stevens et al., 1986) In addition C5a appears to have effects on cytokine release. The effects of C5a in sepsis differ from species to species however and rat and murine models of C5a in sepsis do not mirror the processes seen in human subjects.

The mechanisms by which complement products can elicit impairment of the immune system are not fully understood, however C5a has been shown, in several in vitro studies, to inhibit neutrophil phagocytic function in a dose dependent manner. (Huber-Lang et al., 2002, Conway Morris et al.,) Neutrophils have a large number of C5a receptors (C5aR/CD88) on their cell surface and when C5a is released into the circulation rapid binding of the ligand to the receptor occurs. Once bound the ligand-receptor complex is rapidly internalised resulting in clearance of C5a from the plasma. (Oppermann et al., 1994, Naik et al., 1997). As a result, measurement of serum levels is an inaccurate marker of C5a exposure with low neutrophil CD88 surface expression being a more reliable indicator. Low neutrophil C5aR expression is widely seen in sepsis and has been demonstrated to relate to disease severity and be associated with poor outcomes. (Furebring et al., 2002, Conway Morris et al.,2009, 2013). In addition, this effect has also been seen in individuals with non-septic critical illness including polytrauma. (Amara et al., 2010)

Following internalisation the ligand receptor complex is translocated to the golgi apparatus where under acidic conditions the C5a is hydrolysed and the receptor returned to be expressed on the cell surface once again. (Ward., 2004, Furebring et al., 2002) Re-expression of C5a on the cell surface has been shown to be associated with functional recovery of neutrophil innate immune function (Guo et al., 2004).

A recent study identified a significant positive correlation ($r=0.6904$, $p=0.0004$)

between neutrophil surface CD88 expression and phagocytic capacity supporting the recognized role for C5a in the development of neutrophil functional impairment and the potential of CD88 expression as a biomarker for neutrophil dysfunction in critical illness. (Conway Morris et al., 2009) The first stage of this study will seek to confirm the validity of neutrophil CD88 expression as a biomarker for neutrophil phagocytic capacity within our laboratory and as the final step for eligibility for participation in the trial phases.

Having demonstrated dysfunctional phagocytosis in critically ill patients and having identified C5a as an apparent culprit for this impairment Conway Morris et al sought to investigate mechanisms for restoring phagocytosis in this context.

Conway Morris et al demonstrated that GM-CSF was able to restore phagocytic function in neutrophils from healthy volunteers exposed to C5a in the lab and also in critically ill patients with suspected VAP. In every one of 24 critically ill patients studied, phagocytic function was improved by *ex vivo* application of GM-CSF. (Conway Morris et al., 2009)

1.7 Granulocyte-Macrophage Colony Stimulating Factor

Colony stimulating factors were first described in the 1960s and were named according to their ability to induce colony formation (Gasson et al., 1991). Granulocyte-macrophage colony stimulating factor is a cytokine with haematopoietic growth factor properties acting on the myeloid line of progenitor cells. Compared to G-CSF, GM-CSF has a broader spectrum of leukocyte stimulation. It acts to promote proliferation and differentiation of progenitor cells (Gasson et al., 1991) in the myeloid line in addition to delaying apoptosis (Lopez et al., 1986, Brach et al., 1992) , promoting survival and increasing the functional capacity of mature granulocytes including neutrophils, macrophages and eosinophils (Lopez et al., 1986, Fleischmann et al., 1986, Cebon et al., 1990, Weisbart et al., 1987).

1.7.1 Structure of GM-CSF

GM-CSF is a 23 kDa protein composed of a 4 helical bundle structure. It acts by binding to its heterodimeric receptor on granulocytic and macrophage cells (Metcalf, 2010). Once binding occurs a subsequent cascade of intra-cellular signaling alters

cellular function. Native GM-CSF is a glycoprotein with variable glycosylation (Cebon et al., 1990). The clinical and physiological importance of glycosylation is not fully understood and glycosylation does not appear to be essential for function *in vitro* or *in vivo* (Lieschke et al., 1992), however there is some evidence to suggest that different degrees of glycosylation may influence biological activity including rate of cell proliferation and magnitude of superoxide production (Cebon et al.,1990). Recombinant forms of GM-CSF have been available since the late 1980s following the cloning of the mouse GM-CSF gene in 1984 with cloning of human GM-CSF DNA following in 1985 (Cantrell et al., 1985).

1.7.2 Function of endogenous GM-CSF

Endogenous GM-CSF is a product of many different cells including activated T-lymphocytes, fibroblasts, endothelial cells, macrophages and stromal cells. It may also be produced by B-lymphocytes, mast cells, eosinophils and osteoblasts (Lieschke et al.,1992). Its release is inhibited by anti-inflammatory agents including steroids, and cytokines such as IL-4, IL-13 and IL- 10. (Lenhoff et al., 1998)

GM-CSF is usually present in very low levels in human serum (Presneill et al., 2000, Lieschke et al., 1992) and at these levels is unlikely to have a systemic effect but it probably has an important role when released locally. It appears to be essential in maintaining the function of certain populations of macrophage. GM-CSF knock-out mice and patients with increased levels of anti-GM-CSF antibodies develop significant problems with lung disease due to their inability to clear surfactant (in humans the resulting disease is known as pulmonary alveolar proteinosis). In addition, these patients have increased mortality from bacterial infections and have been demonstrated to have impaired neutrophil function (Uchida et al., 2007). Reduced levels of endogenous GM-CSF have been reported in sepsis and associated with poor outcomes (Perry et al., 2002).

Elevation of GM-CSF locally is seen in some inflammatory conditions such as multiple sclerosis and rheumatoid arthritis (Xu et al., 1989) where it may have harmful effects and contribute to the pathogenesis of these conditions (Williamson et al., 1988).

1.7.3 Effects of exogenous GM-CSF

In the 1980s following the cloning of the genes responsible for both murine and human GM-CSF the production of large amounts of these recombinant factors enabled more detailed studies of the effects of GM-CSF. Up until this point researchers had only been able to extract small amounts of endogenous GM-CSF with limited results.

When GM-CSF is administered both *in-vitro* and *in-vivo* it exerts multiple effects on cells of the myeloid lineage by increasing the proliferation and differentiation of progenitor cells whilst also stimulating the functional activities of mature cells including neutrophils, eosinophils, monocytes and macrophages (Rapoport et al.,1992, Lopez et al., 1986). In contrast to G-CSF the increase in proliferation of progenitor cells is more modest while greater effects are seen on the function of mature myeloid cells (Fischmeister et al., 1999).

The effects of GM-CSF on neutrophils are both direct (prolonging survival and promoting degranulation) (Rapoport et al.,1992, Gasson et al., 1984) and indirect (enhancing phagocytosis, bacterial killing, respiratory burst and calcium flux), (Rapoport et al.,1992, Weisbart et al., 1985). *In vitro* studies have demonstrated enhanced phagocytosis (Fleischmann et al., 1986, Lopez et al., 1986, Conway Morris et al.2009) and superoxide release (Weisbart et al., 1985; Lopez et al, 1986) when GM-CSF is applied to neutrophils.

Effects on survival and delayed apoptosis of neutrophils appear to be due to inhibition of DNA fragmentation and the production of new RNA and proteins. *In vitro* experiments have demonstrated increases of 25 to 100% in neutrophil survival time when GM-CSF is added to incubating medium. (Brach et al.,1992)

1.7.4 Pharmacokinetics of GM-CSF

Recombinant human GM-CSF can be administered by the subcutaneous and intravenous routes. *In- vitro* studies using human bone marrow cells have shown a dose-dependent response in terms of progenitor cell proliferation (Grabstein et al., 1986), and an increase in chemotaxis and anti-fungal and anti-parasitic activities of neutrophils and monocytes (Reed et al., 1987, Reed et al., 1990). Concentrations ranging from 1-100ng/ml have been studied. Pharmacological and toxicological studies carried out on monkeys showed no major visceral organ pathology following

either single dose or daily dosing up to one month by either intravenous or subcutaneous preparations using doses up to 200 micrograms/kg/day. As with *in vitro* human studies dose dependent responses were seen in terms of leucocyte proliferation, with counts recovering to baseline within 1 -2 weeks (Sanofi-Aventis, 2013). Human *in-vivo* pharmacokinetics of GM-CSF have had limited investigation and in particular there are little data showing the pharmacokinetics of GM-CSF in critically ill patients (Cebon., 1988). Several studies have been performed in healthy subjects and in patients with cancer although results appear to vary. Subcutaneous administration is associated with lower and less rapid peak levels (Cebon et al., 1988, Honkoop et al., 1996, Hovgaard et al., 1993), and probably as a consequence a reduced level of adverse reactions (Honkoop et al., 1996, Lieschke et al.,1989). When Leukine (sargramostim, yeast derived GM-CSF) was administered subcutaneously to healthy volunteers it was detectable in the blood at 15 minutes, peak concentrations occurred between 1 and 3 hours and it remained detectable for up to 12 hours post injection depending on the dose(Cebon et al., 1988,Hovgaard et al.,1993; Sanofi-Aventis, 2013).There is evidence to suggest that there is a lower rate of adverse effects seen with yeast-derived GM-CSF compared to Ecoli-derived GM-CSF and for this reason yeast derived product has been selected for use in this study (Dorr 1993).Published pharmacokinetic studies report variable serum levels, in part related to dose and route of administration. The efficacy of GM-CSF appears to correlate with the length of time for which serum levels are maintained above 1ng/ml (Cebon et al., 1988). Following subcutaneous administration serum levels >1ng/ml can be achieved for up to 16 hours (Honkoop et al., 1996). It is likely that in critically ill patients absorption may be impaired although clinically immune cell function responses have been seen with doses of 3-4 microgrammes per kilogram in previous clinical trials (Presneill et al., 200, Meisel et al., 2009,Orcozo et al.,2006).

1.7.5 Metabolism and clearance

The metabolism of GM-CSF is not fully understood. It is thought to occur within the circulation following receptor binding and very low levels of GM-CSF (0.001%-0.2% of the injected dose) have been detected in the urine in previous studies (Cebon., 1990). Pre-treatment levels of serum GM-CSF are reached between 6 and 20 hours after injection depending on the dose and route of administration (Hovgaard et al.,

1993).

1.7.6 Clinical Experience with GM-CSF

1.7.6.1 License

Sargramostim, a commercially available human recombinant GM-CSF is manufactured in the US under the trade name Leukine. It is a glycoprotein of 127 amino acids produced through the mechanisms of recombinant DNA technology in a *Saccharomyces cerevisiae*-based system. Leukine differs structurally from natural human GM-CSF with an alteration in its amino acid sequence - substitution of leucine at position 23 (Genzyme, 2009).

Leukine / sargramostim is currently licensed in the United States of America for restoration of the myeloid cell line number post chemotherapy and bone marrow transplant. (Genzyme, 2009, Mehta et al., 2015) It is used in the treatment of older (55-70 years) neutropaenic patients with acute myeloid leukaemia post-chemotherapy where it hastens neutrophil recovery and reduces the incidence of, and death rate from, serious infections (Rowe et al.,1995, Mehtaet al., 2015).

1.7.6.2 Indications for GM-CSF

1.7.6.2.1 Post chemotherapy and bone-marrow transplant

As described above GM-CSF hastens recovery from neutropaenia in patients post – chemotherapy. Studies of GM-CSF in the treatment of patients post autologous and allogenic bone marrow transplant show significant improvements in the rate of myeloid engraftment, duration of hospital stay and frequency of infections (Nemunaitis., 1991, Powles et al., 1990). A shorter duration of antibiotic therapy has also been reported in those patients receiving Leukine post bone marrow transplant (Nemunaitis et al., 1991).

1.7.6.2.2 Sepsis

The largest area of literature in the use of GM-CSF in sepsis relates to the care of neutropaenic premature babies. It has been demonstrated to enhance neutrophil recovery and possibly reduces nosocomial infection rates but has no impact on

survival (Carr et al., 2009, Bilgin et al., 2001).

Few studies have looked at the use of GM-CSF in the treatment of non-neutropaenic septic adults, however some recently published studies have shown beneficial effects in terms of various parameters of sepsis-related immune dysfunction in addition to non-significant benefits in clinical outcomes.

A double-blind randomised controlled trial of 38 patients with severe sepsis or septic shock and evidence for immunosuppression, as indicated by low monocyte HLA-DR expression, were randomised to receive treatment with either subcutaneous GM-CSF (4mcgm/kg/day) or placebo for 8 days. The primary outcome was recovery in monocyte HLA-DR expression. Patients whose monocyte HLA-DR levels failed to recover to the normal range had their dose increased to 8mcg/kg/day at day 5. As well as demonstrating a statistically significant difference in the primary outcome, significant reductions were seen in the duration of mechanical ventilation and APACHE II score. A trend towards a reduction in ICU and hospital stay was also observed (Meisel et al.,2009).

Another double-blind RCT of nineteen patients, all with severe sepsis and sepsis-associated respiratory organ dysfunction, randomised participants to receive either intravenous GM-CSF 3mcgm/kg/day or placebo for 5 days. Concerns have previously been raised regarding the potential of GM-CSF to worsen adult respiratory distress syndrome (ARDS). Although the investigators found no difference in their primary outcome of mortality, a significant improvement in PaO_2:FiO_2 was seen accompanied by a trend towards a reduction in ARDS in those patients treated with GM-CSF (Presneill et al.,2002). Increased alveolar levels of GM-CSF have previously been found to be associated with improved survival from ARDS (Mateute Bello et al 2000).

Orozco et al published a trial looking at the use of subcutaneous GM-CSF following surgery for abdominal sepsis. Again they randomised patients to receive either GM-CSF or placebo finding a significant improvement in the rate of recovery, and reductions in length of stay and wound infection in those treated with GM-CSF (Orcozo et al.,2006).

A trial by Rosenbloom et al produced similar positive results including quicker resolution of infection in their cohort of septic patients following intravenous infusion of GM-CSF for 72 hours. This study, however, recruited a significant proportion of

solid organ transplant patients on systemic immunosuppression and so the results are difficult to extrapolate to the ICU population as a whole (Rosenbloom et al.,2005).

No published trials have studied the use of GM-CSF in patients with demonstrated neutrophil dysfunction. It is possible that with such targeted intervention results may be more significant.

1.7.6.2.3 Other Indications for GM-CSF

The potential immunomodulatory properties of GM-CSF have been investigated in relation to a wide spectrum of disease including prostate cancer (Amato et al.,2009, Small et al., 1999), Crohn's disease (Korzenik et al., 2005), human immunodeficiency virus (HIV) infection (Brites et al., 2000), childhood neuroblastoma (Yu et al., 2010) and for use in vaccination strategies (Dranoff.,2002). Inhaled GM-CSF is currently used in the treatment of patients with pulmonary alveolar proteinosis (PAP) where the presence of anti-GM-CSF antibodies results in accumulation of surfactant and respiratory failure (Wylam et al.,2006). The literature also reports beneficial topical use of GM-CSF for the treatment of burns (Zhang et al., 2009) and cervical intraepithelial neoplasia (Hubert et al.,2010). However, with the possible exception of prostate cancer and PAP, so far there is insufficient evidence to recommend widespread application of GM-CSF in these settings.

1.7.7 Safety profile and side effects

Recombinant GM-CSF is used in various areas of clinical practice. The emerging consensus is that GM-CSF demonstrates low toxicity when used in doses of less than 10mcgm/kg/day. Some concerns have been highlighted in relation to the administration of GM-CSF to various patient groups due to the potential for GM-CSF to exacerbate underlying disease or development of serious side effects. In particular, there are concerns in relation to its use in the following patient groups; haematological malignancies, autoimmune diseases, some solid organ cancers and multiple sclerosis.

The drug is contraindicated in any patient with a known hypersensitivity to GM-CSF,

any patient with >10% blast cells in the bone marrow or peripheral blood and any patient who has received chemotherapy or radiotherapy in the previous 24 hours. (Sanofi-Aventis, 2013).

The side effects of GM-CSF are in part mediated by the production of secondary cytokines including TNF-α and IL-6. Common side effects include fever, pain and erythema at the injection site and generalized bone pain and myalgia (Lieschke et al., 1989, Sanofi-Aventis, 2013). Dose-dependent abnormalities in liver enzymes and renal function have been seen. (Lieschke et al., 1989, Honkoop et al.,1996, SmPC, 2011). Thrombotic complications have been reported and are probably related to the production of TNF in turn triggering the clotting cascade (Honkoop et al., 1996, Sanofi-Aventis, 2013). A ‘first dose syndrome’ is described characterized by flushing, tachycardia, dyspnea, hypotension and respiratory distress requiring symptomatic treatment only and resolving spontaneously with no recurrence on repeated administration (Lieschke et al., 1989, Sanofi-Aventis, 2013).

1.8 Hypothesis and aims

The aim of this work was to investigate the hypothesis that subcutaneous GM-CSF can restore effective neutrophil function in critically ill patients in whom it is know to be impaired.

I initially carried out an assay confirmation study, designed with the aim of validating the use of neutrophil CD88 expression as a biomarker for neutrophil phagocytic impairment. If shown to be a reliable marker, then it would be suitable for use in identifying patients eligible for both the dose finding study and the randomized controlled trial.

On completion of the assay confirmation study I undertook the dose finding study which aimed to establish the optimum dose and duration of GM-CSF which would be both effective in terms of restoring neutrophil phagocytic capacity and safe within the patient cohort.

Finally, informed by the results of both the assay confirmation study and the dose finding study I carried out a randomised controlled trial to answer the question of whether GM-CSF could restore effective neutrophil function in critically ill patients. In completing this work I took the opportunity to examine several different secondary laboratory and clinical outcomes relevant to the clinical problem in question. The results of this work are described in the chapters which follow.

Chapter 2: Materials and Methods

As previously described this research project was composed of 3 phases, comprising an initial Assay Confirmation Study, a Dose Finding Study and finally a Randomised Controlled Trial. This chapter will outline the design and conduct of each phase of this work and describe the materials and methods used.

2.1 Design of the study

2.1.1 Assay Confirmation Study

The Assay Confirmation Study (ACS) was an observational study designed to locally validate the use of neutrophil surface CD88 (C5aR) expression as a surrogate biomarker for impaired neutrophil phagocytic function. As described earlier (Chapter 1, section 1.6.1) previous research from our group had demonstrated that CD88 expression on neutrophils was a reliable surrogate marker for phagocytic capacity (Conway Morris et al 2009).

2.1.1.1 Aim of the study

The aim of the ACS was to identify a threshold level of CD88 expression, within our local laboratory, which would correspond to a phagocytic capacity of 50% and serve as a cut-off for final eligibility for participation in the subsequent phases of the research. Concurrently samples from a pool of unmatched healthy volunteers underwent the same analysis.

2.1.1.2 Conduct of the study

This phase of the study was designed to recruit up to 30 patients, all of whom fulfilled the criteria for entry into the dose finding study (DFS) and randomised controlled trial, and each of whom would undergo blood sampling for paired measurement of neutrophil CD88 expression and neutrophil phagocytic capacity. Only basic demographic data (age and sex) were collected on these patients. No alterations in clinical care were made. Participation in the study completed following collection of a single blood sample.

2.1.1.3 Schematic diagram of study design

The schematic diagram below outlines the design of the Assay Confirmation Study.

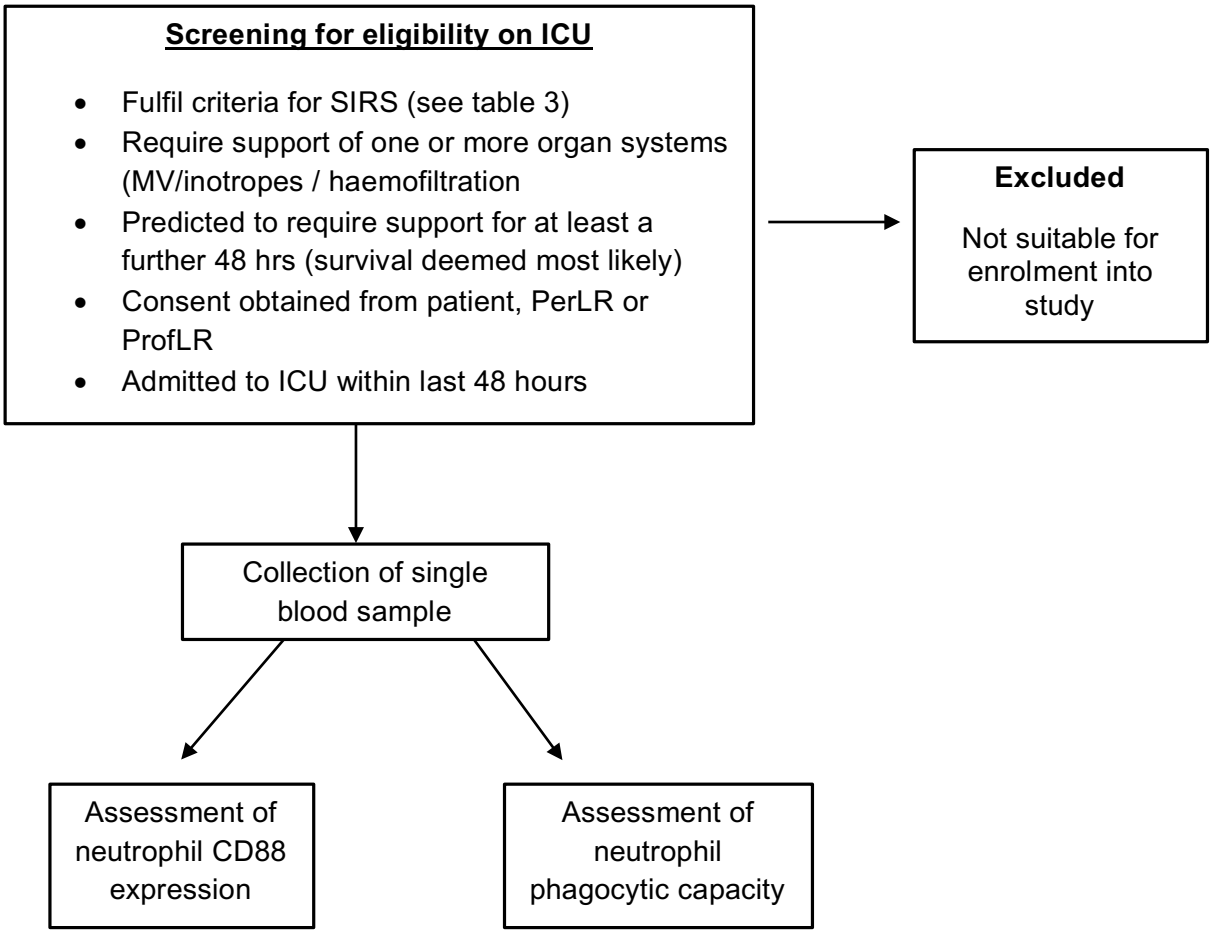

Figure 2a: Schematic diagram of assay confirmation study procedures

Systemic inflammatory response syndrome criteria (SIRS)
• Temperature < 36 or >38 °C
• White blood cell count <4x10^9/L or >12x109/L or 10% Band
• Heart rate > 90 beats per minute
• Tachypnoea with respiratory rate > 20 breaths per minute or $PaCO_2$ < 4.3kPa

Table 3: Criteria for the systemic inflammatory response syndrome (SIRS)

2.1.2 Dose Finding Study (DFS)

The Dose Finding Study was an open labelled study designed to to compare 2 doses of GM-CSF administered for 2 different durations of treatment. The study planned to recruit up to a maximum of 24 patients in 4 separate dosing sub-groups and compare their response to treatment in terms of improvement in phagocytosis.

2.1.2.1 Aim of the study

The aim of the DFS was to establish the optimum dose and duration of GM-CSF (that which would produce a biological response in terms of improvement in phagocytosis alongside a favourable safety profile) which would then be carried forward for use in the RCT.

2.1.2.2 Conduct of the study

This phase of the study was designed to recruit patients who would fulfil the criteria for entry into the subsequent RCT. Following consent blood would be collected from patients to assess their neutrophil phagocytic capacity as the final step in their eligibility for the DFS. This would be known as the baseline or 'day 0' blood sample. Neutrophil phagocytic capacity was measured as the percentage of isolated neutrophils ingesting 2 or more zymosan particles following incubation with a zymosan 'meal'. (chapter 2, section 2.16.3). Those patients with confirmed neutrophil phagocytic capacity <50% would continue in the DFS and receive their first daily dose of GM-CSF later that day (day 0). GM-CSF would be continued for 4 or 7 days in total depending on the participant's sequence in recruitment as outlined below.

Patient enrolment number	Dose of Leukine (Sargramostim, rhu GM-CSF)
1-6	3μg/kg/day for 4 days
7-12	3μg/kg/day for 7 days
13-18	6μg/kg/day for 4 days
19-24	6μg/kg/day for 7 days

Table 4: Drug allocation schedule for dose finding study

2.1.2.3 Schematic diagram of study design

The schematic diagram below outlines the design of the Dose Finding Study.

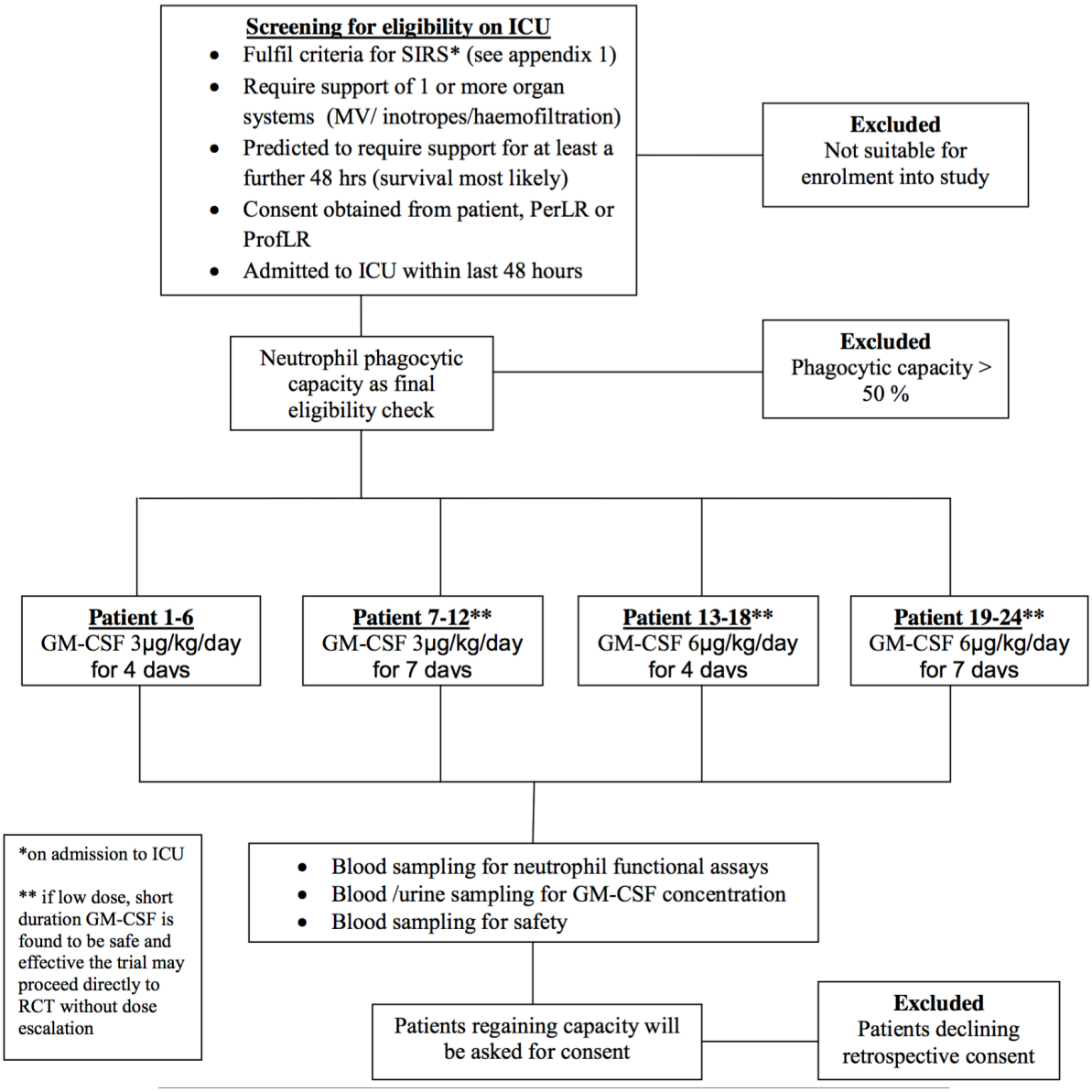

Figure 2b: Schematic diagram of the dose finding study procedures. For SIRS criteria see table 3.

2.1.2.4 Trial-related procedures

Blood samples would be collected on all study days up to day 9 for assessment of neutrophil function, monocyte HLA-DR expression and safety. The table below outlines the schedule of trial-related procedures for the Dose Finding Study.

		Day									
	-1	0	1	2	3	4	5	6	7	8	9
Eligibility assessment	X										
Informed Consent	X										
Neutrophil phagocytic capacity quantification	X										
Study drug administration		X	X	X	X	X*	X*	X*			
Blood sampling (safety ≤10mls)		X	X	X	X	X	X	X	X	X	X
Blood sampling (phagocytosis ≤ 20mls)		X	X	X	X	X	X	X	X	X	X
Neutrophil CD88 /monocyte HLA-DR expression/ GM-CSF concentration		X	X	X	X	X	X	X	X	X	X
Adverse events		X	X	X	X	X	X	X	X	X	X

Table 5. Trial related procedures in dose finding study

2.1.2.5 Dose escalation scheme

The study was designed to recruit 6 patients to each dosing schedule. To ensure the safety of participants at all times it was agreed that within each dose cohort the first patient to be recruited would be treated and observed for 3 days, after the initial injection of subcutaneous GM-CSF, prior to open recruitment commencing. Safety and tolerability data would be collected during the treatment and observation period for all six patients in the cohort. Prior to dose escalation taking place these data

would be collated and presented to the Data Monitoring and Safety Committee (DMSC). In the event of 2 or more of the 6 patients, treated within a dosing cohort, being found to have experienced a dose-limiting toxicity dose escalation would not take place. The final decision regarding dose escalation would be made by the DMSC.

2.1.2.6 Dose limiting toxicity

A dose-limiting toxicity was described as *'any serious adverse event (SAE) which is judged to be probably or definitely related to the administration of GM-CSF.'* (Study protocol, appendix A). Suspected adverse events would be recorded in line with the trial protocols for pharmacovigilance. Following administration of the first dose of GM-CSF blood would be collected each morning for assessment of neutrophil phagocytic capacity, CD88 expression, monocyte HLA-DR, and safety analysis including full blood count, biochemical profile and liver function tests. Post-dose blood samples were collected for assessment of serum levels of GM-CSF.

2.1.2.7 Selection of optimum dose for RCT

In selecting the dose of GM-CSF which would be carried forward for use in the RCT we planned to evaluate both the efficacy (the ability of the drug to produce a demonstrable increase in neutrophil phagocytic capacity) and the tolerability (i.e. <2 of the 6 patients treated in the cohort experiencing a dose-limiting toxicity) of each combination of dose and duration of GM-CSF.

2.1.3 Randomised controlled trial

The randomised controlled trial was designed to test the hypothesis that clinical administration of GM-CSF in critically ill patients with known neutrophil dysfunction would replicate the effects seen *ex-vivo* in the blood of critically ill patients by restoring effective neutrophil phagocytosis.

2.1.3.1 Aim of the study

The primary outcome of the RCT was

- Neutrophil phagocytic capacity 2 days after administration of GM-CSF/placebo (as measured by the percentage of neutrophils ingesting ≥2 zymosan particles *ex-vivo,* chapter 2, section 2.16.3).

Secondary outcomes included

- Sequential neutrophil phagocytic capacity on alternate days (to determine sustainability of any observed effects in the primary outcome measure)
- Neutrophil phagocytic capacity measured as 'area under the curve' over the study period
- Other assessments of neutrophil function (including reactive oxygen species generation, migratory capacity and apoptotic rate)
- Monocyte HLA-DR expression
- Sequential Organ Failure Assessment (SOFA) score (Vincent et al., 1998)
- Length of stay in ICU
- Incidence of ICUAIs (as defined by the Hospitals in Europe Link for Infection Control Surveillance (HELICS) criteria (Mertens et al. 1996)
- All cause mortality 30 days post randomisation
- Number of days of mechanical ventilation to day 30.

2.1.3.2 Conduct of the study

In line with the DFS, on the morning following screening and obtaining consent, blood was drawn for assessment of neutrophil phagocytosis (by incubation of isolated neutrophil with a zymosan 'meal', section 2.16.3) as the final step in assessment of eligibility for entry into the trial. If the neutrophil phagocytic capacity was found to be less than 50% the patient would continue in the trial and would undergo double-blind randomisation to receive a daily injection of either GM-CSF (at a dose of 3μg/kg/day or 6μg/kg/day) or placebo for 4 or 7 days depending on the outcome of the DFS.

During the RCT blood was drawn for assessment of neutrophil phagocytosis, neutrophil functional assays, monocyte HLA-DR expression and other tests of inflammation on alternate study days up until day 9. Blood was drawn on a daily basis for safety assessment (these samples formed part of usual clinical care).

2.1.3.3 Schematic diagram of study design

The schematic diagram below outlines the design of the randomised controlled trial.

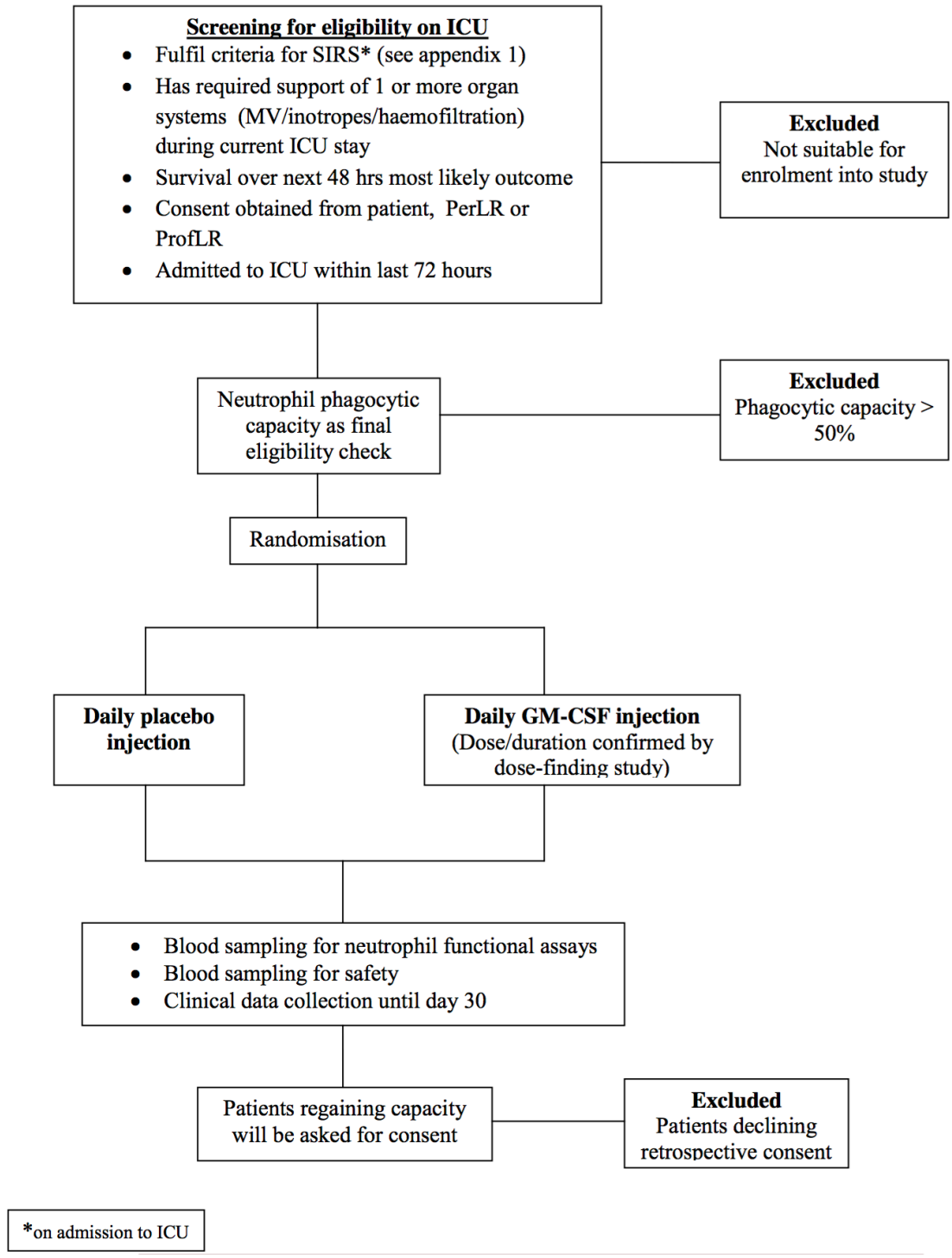

Figure 2c: Schematic diagram of the randomized controlled trial procedures

2.1.3.4 Trial-related procedures

During the RCT blood samples would be collected at baseline, day 2 and alternate study days up to day 9 for assessment of neutrophil function, monocyte HLA-DR expression and Blood samples would be collected on all study days up to day 9 for assessment of safety. The table below outlines the schedule of trial-related procedures for the randomized controlled trial.

		Day										
	-1	0	1	2	3	4	5	6	7	8	9	10-30
Eligibility assessment	X											
Informed consent	X											
Neutrophil CD88 quantification		X		X		X**		X**		X**		
Randomisation		X										
Study drug administration		X	X	X	X	X*	X*	X*				
Blood sampling (safety ≤10ml)		X	X	X	X	X	X	X	X	X	X	
Blood sampling (phagocytosis ≤ 20ml)		X		X		X		X		X		
SOFA score		X	X	X	X	X	X	X	X	X	X	X
ICU status (i.e. whether still in ICU)		X	X	X	X	X	X	X	X	X	X	X
Adverse events		X	X	X	X	X	X	X	X	X	X	
Survival analysis		X	X	X	X	X	X	X	X	X	X	X
ICUAI (by HELICS criteria)		X	X	X	X	X	X	X	X	X	X	X
Neutrophil functional assays, monocyte HLA-DR expression ≤5mls		X**		X**		X**		X**		X**		

Table 6: Trial procedures for randomised controlled trial. * Duration of administration of study drug to be decided following outcome of DFS. ** Blood samples collected on alternate days during RCT- day 4 or 5, day 6 or 7, day 8 or 9 depending on availability of research laboratory staff. HELICS, hospitals in Europe Link for infection control and surveillance; HLA-DR, human leucocyte antigen–antigen D related; ICU, intensive care unit; SOFA, sequential organ failure assessment;

2.2 Regulatory approvals

Prior to commencing the studies, the necessary approvals were sought from the appropriate regulatory authorities.

Ethical Approval was granted by the Leeds West National Ethics Committee (REC Ref: 12/YH/0083). This committee were specialised in the assessment of Clinical Trials of Investigational Medicinal Products (CTIMPs) and flagged for trials involving Adults Lacking Capacity. This requires the ethics committee to have a member with professional experience of the disease and patient population concerned or to co-opt such a member from another REC or to seek advice from an independent professional expert. Ethical approval for all 3 studies was granted at the outset.

As this study was a Clinical Trial of an Investigational Medicinal Product approval was also sought from and granted by the Medicines and Healthcare Technology Regulatory Authority (MHRA).

The relevant Research and Development (R&D) departments at each of the participating sites granted local approval for the study to take place namely, Newcastle upon Tyne Hospitals NHS Foundation Trust (Sponsor R&D – Royal Victoria Infirmary and Freeman Hospital sites) and Queen Elizabeth Hospital Gateshead R&D. Both of these hospitals took part in all 3 phases of the trial.

Approval was received from the Ethics Committee, the MHRA and the local R&D department for Sunderland Royal Hospital to join the trial as an additional site for the Randomised Controlled Trial.

2.3 Clinical trial registration

The trial was registered with the European Clinical Trials Database (EudraCT No: 2011-005815-10) and International Standard Randomised Controlled Trial Number (ISRCTN: 95325384). All trial procedures were carried out to Good Clinical Practice standards.

The study was also registered with the INVOLVE open-access database (http://www.involve.org.uk), part of the National Institute for Health Research, which supports active public involvement in NHS research. During the design phase of the study the protocol for both the DFS and RCT was shared with members of CritPaL

(The Patient Liaison Committee of the Intensive Care Society) for their comments and feedback.

2.4 Sponsorship and Funding

The trial was sponsored by Newcastle upon Tyne Hospitals NHS Foundation Trust and was funded by the Medical Research Council through the Developmental Clinical Studies scheme (Grant No: G1100233).

2.5 Study Management

The study was managed by Newcastle Clinical Trials Unit, with 2 trial managers allocated (one at any given time) over the course of the study (Ms Melinda Jeffels and Mrs Jennie Parker). The trial steering committee / project management group comprised of the chief investigator, the principal investigators at each site, the clinical research associate and the trial managers convened at regular intervals during the set-up and course of the trial.

2.6 Consent for participation

Where possible, consent for participation in the trial was obtained from the individual concerned. Because of the nature of the trial, in the vast majority of cases the participant did not have capacity to give informed consent. In these circumstances consent was obtained from a close relative or friend (Personal Legal Representative, PerLR) or where no such person existed a Professional Legal Representative (PerLR, a doctor not connected with the conduct of the trial, who was responsible for the patient's care). Because of the fact that eligibility for recruitment to the trial included some time dependent factors patients or their PerLR / ProfLR were asked to provide a decision regarding consent within six hours if they felt able to do so. A period of up to 24 hours was offered to those making a decision regarding provision of consent where this was requested.

The law surrounding consent for Adults Lacking Capacity participating in Clinical Trials of Investigational Medicinal Products (CTIMPs) differs from that of non-CTIMPs and is set out in the Medicines for Human Use (Clinical Trials) Regulations 2004. One of the key differences is that for adults lacking capacity informed consent is

given by the legal representative (rather than assent in the case of non-CTIMPs) and is considered to represent the subjects presumed will. The threshold for carrying out CTIMPs including Adults Lacking Capacity is higher than that for those with capacity and relies on several principles. The trial must relate directly to the condition with which the patient is suffering, there must be a clear expectation that the potential benefit of the IMP in question must outweigh any potential risks and the trial must be necessary to validate data already collected which could not be validated further without including such a population. Where possible the individual concerned should be involved as far as possible in the decision making process being provided with information appropriate to their level of capacity. In line with the guidelines for gaining consent in such circumstances the participants or their PerLR /Prof LR were informed that consent could be withdrawn at any time without prejudice.

Once a participant's capacity had been deemed to have returned retrospective consent was sought in all cases. Where consent to continue in the trial was not given the participant was withdrawn from the trial at that stage. The participant was then asked whether they gave consent for the data already collected to be used for the purposes of the research.

2.7 Screening and recruitment

2.7.1 Critically ill patients (ACS/DFS/RCT)

Screening took place of all patients on each intensive care unit on a daily basis from Monday to Friday inclusive. Patients were eligible for participation in all phases of the study if they met the following criteria:

To ensure patient safety in this proof of concept study a comprehensive list of exclusion criteria was put in place aiming to minimise the risk of any potential harm.

Inclusion criteria
• Fulfil the criteria for SIRS (see section 2.1.1) • Requires the support of 1 or more organ system (mechanical ventilation, inotropes, haemofiltration) • Predicted to require support for at least a further 48 hours (survival most likely outcome) • Admitted to ICU within last 48 hours.
Exclusion criteria
• Absence/refusal of informed consent • Current prescription of a colony stimulating factor • Any history of allergy/adverse reaction to GM-CSF • Total white cell count >$20x10^9$/litre at time of screening • Haemoglobin < 8.5g/dl at the time of screening • Age < 18 years • Pregnancy or lactation • Known in-born errors of neutrophil metabolism • Known haematological malignancy and/or known to have >10% peripheral blood blast cells • Known aplastic anaemia or pancytopenia • Platelet count <$50x10^9$/litre • History of cancer (unless curative resection / treatment performed) • Chemotherapy or radiotherapy within the last 24 hours • Solid organ or bone marrow transplantation • Use of maintenance immunosuppressive drugs other than maintenance corticosteroids (allowed up to 10mg prednisolone/day or equivalent) • Known HIV infection • Active connective tissue disease (e.g. rheumatoid disease, systemic lupus erythematosus) requiring active pharmacological treatment. • ST-segment elevation myocardial infarction, acute pericarditis (by ECG criteria) or pulmonary embolism (radiographically confirmed) in previous week • Involvement in any study involving an investigational medicinal product in the previous 30 days

Table 7: Exclusion criteria for ACS, DFS and RCT

If all inclusion criteria were met and there was no evidence to exclude participation, the patient and/or their family were approached by a member of the trial team and provided with a full verbal explanation of the study and a copy of the patient information sheet. The patient and/or their PerLR were then given time to consider the study and a member of the research team returned to answer any further questions. As discussed because of the time-dependent nature of the study patients or their legal representatives were given up to 24 hours to make a decision regarding consent but were asked to make a decision within 6 hours if they felt able.

Signed consent was taken from the relevant individual and a copy of the consent form given to the patient / PerLR / ProfLR, filed in the patient's notes and stored in the site file. In the event that the patient recovered capacity the law states that the consent given by the legal representative stands but the patient should be consulted and their views respected. Retrospective consent was sought from all patients who regained capacity during the course of the study.

2.7.2 Healthy volunteers (ACS only)

Healthy volunteers were recruited from the university staff and student population. Healthy volunteers, 18 years of age and older, were invited to participate via posters displayed on university noticeboards and via email within our research institute.

Healthy volunteers responding to the information were provided with a participant information sheet outlining the purpose of the study, the risks and benefits of taking part, the exclusion criteria and the procedure for collection of their blood sample. Healthy volunteers were excluded from participation if any of the following circumstances existed

I. the volunteer had donated blood in the previous 90 days (either to the Blood Transfusion Service or to any research study)
II. the volunteer had donated more than 1 litre of blood in total over the previous 12 months
III. the volunteer had been anaemic at any time in the last 12 months
IV. the volunteer was on any regular medication (oral contraceptive pill was permissible in female patients)

Once a healthy volunteer had been deemed eligible to participate, and had been given ample opportunity to read and consider the patient information sheet, written consent was obtained.

Following donation of the blood sample each volunteer was offered a £5 book token in recognition of the time taken and inconvenience experienced in participating in the study.

2.8 Assessment of baseline phagocytic capacity (DFS/RCT)

As the final assessment of eligibility for participation in both the DFS and RCT blood was drawn for baseline assessment of neutrophil phagocytic capacity. If the neutrophil phagocytic capacity, as measured by the percentage of isolated neutrophils ingesting 2 or more zymosan particles ex-vivo, was less than 50% the patient continued in the study. If the patient demonstrated adequate phagocytic capacity (phagocytosis ≥50%) they were excluded from the study at this stage.

2.9 Randomisation (RCT)

Following assessment of the neutrophil phagocytic capacity those patients with neutrophil phagocytic capacity <50% were to be randomised in a double-blind fashion to receive either IMP (GM-CSF) or placebo. A member of the study team with delegated responsibility for randomisation entered the patient's initials, date of birth and site into an online randomisation system managed by the Newcastle Clinical Trials Unit. The site was password protected and accessible only to designated members of the clinical trial team. Randomisation was performed in a 1:1 ratio. An independent statistician was responsible for determining the randomisation allocation schedule. The allocation schedule was stratified by site and designed to contain permuted blocks of variable length in order to minimise the risk of breach of concealment. The randomisation schedules were held within the clinical trials pharmacy at each site.

Following generation of the participant randomisation number via the online programme a prescription including the randomisation treatment number was completed by a member of the trial team and delivered to the clinical trials pharmacy from where the IMP/placebo was issued.

2.10 Blinding (RCT)

The DFS was designed to be run as an open label study with all participants receiving the IMP (GM-CSF). Participants, researchers and the usual clinical team were all aware of the prescription and participation in the DFS at all times.

The RCT was designed to be run as a double blinded study with the participants, researchers and usual clinical team intended to be blinded to allocation at all times. Laboratory measures were completed by clinical and laboratory staff who were fully blinded and had no access to allocation information at any time. All study results, including laboratory outcomes and clinical outcomes, were presented to the independent statistical team in a blinded manner and treatment allocation was not revealed until after the independent statistical team had completed their analysis of all of the data.

During the course of the study a protocol was in place for the mechanism by which to unblind the clinical and/or research team should the need arise.

Several technical and logistical aspects of the trial mandated that the reconstitution and administration of the IMP was required to be an unblinded task. Members of the research nursing team were specifically trained to complete this. The following factors contributed to the need for this:

i) The cost of a pre-manufactured, matched placebo was prohibitively expensive (i.e. outside the funding envelope in the grant). Therefore, normal saline was used as placebo on the basis that at the point of administration, normal saline and GM-CSF were identical in appearance (a clear, colourless liquid).

ii) Due to its short shelf life once reconstituted (6 hours), the drug could not be prepared by the clinical trial pharmacy, as no staff were available at weekends. It was therefore necessary for it to be prepared by research nurses just prior to administration.

iii) Due to limited resources and budget for staff costs, and the need to be able to deliver the IMP seven days a week at short notice, it was not possible to recruit a separate unblinded team of research nurses from the local clinical research network.

The only personnel to be unblinded at any stage in the course of the RCT were the research nursing staff involved in the reconstitution and administration of the study drug. All other clinical and research staff, the patient and their relatives remained fully blinded to the treatment allocation at all times.

2.11 Investigational medicinal product (DFS / RCT)

The investigational medicinal product (IMP) used in the DFS and RCT was Sargramostim (Leukine, recombinant human GM-CSF). Recombinant GM-CSF is clinically available in 2 forms; as a glycosylated compound derived from a yeast-based system and as a non-glycosylated compound prepared in an *E. coli*-based system. The two compounds have broadly comparable clinical effects. However, the yeast-derived product has been shown to demonstrate a superior safety profile and it was on this basis that it was selected for use in the DFS and RCT.

2.11.1 Sourcing of IMP (DFS / RCT)

Sargramostim is owned by Genzyme (a subsidiary of Sanofi-Aventis). It has been approved by the FDA and is widely used for the restoration of myeloid cell populations in cancer patients following chemotherapy or bone marrow transplant. In addition to stimulating myeloid cell recovery it has a role in reducing the risk of serious infection in such patients.

It is manufactured in the US to GMP requirements using recombinant DNA technology in a *Saccharomyces cerevisiae*-based system. It is marketed under the trade name Leucine.

As the drug is not licensed in the EU a Qualified Persons Certificate was provided by the drug importing company (Mawdsley Brooks and Co., Quest 22, Quest Park, Doncaster), confirming that the drug had been manufactured to GMP standards. In addition, an Importer's Marketing Authorisation was supplied as required by the MHRA.

The drug was labelled by Mawdsley Brooks and Co. in compliance with the Annex 13 Guidelines regarding investigational medicinal products for use in clinical trials (European Commission, 2010).

2.11.2 Storage of IMP (DFS / RCT)

Stocks of IMP/placebo were stored in the clinical trial pharmacy at each participating site according to the manufacturer's instructions. Following issue of the IMP/placebo, to a research nurse with designated responsibility for the unblinded reconstitution and administration of the study drug, it was transported in a black bag contained within a sealed container to the locked fridge at the ICU site. The IMP /placebo was stored between 2-8° C in a fridge with alarmed continuous temperature monitoring. The fridge was locked at all times and only accessible to members of the research nursing team with responsibility for administration of the IMP.

2.11.3 Reconstitution of IMP (DFS / RCT)

During the DFS GM-CSF was reconstituted in an open-label, unblinded fashion with members of both the usual clinical nursing team and the research nursing team being involved in this task.

During the RCT reconstitution of the IMP/drawing up of placebo was performed by 2 trained, unblinded members of the research nursing team within a locked location away from the patient's bedside and out of sight of all blinded members of the research team and the usual clinical care team. Sargramostim (GM-CSF) was presented as a white lyophilized powder for reconstitution. Each vial of Sargramostim contained 250μg of GM-CSF and was reconstituted with 1ml of sterile water. The IMP/placebo was reconstituted to a final volume, calculated according to weight in weight ranges to the nearest 5kg. The clear, colourless liquids were indistinguishable and unidentifiable as IMP or placebo at the point of administration to ensure maintenance of blinding. The syringe containing IMP/placebo was labelled with the patient's name, date of birth and study number and the final volume of IMP/placebo before being taken to the bedside. In all circumstances, once reconstituted the drug was required to be used within 6 hours.

2.11.4 Administration of the IMP / placebo (DFS / RCT)

During the DFS, following reconstitution, GM-CSF was administered in an open-label, unblinded fashion with members of both the usual clinical nursing team and the research nursing team being involved in this task.

During the RCT, following reconstitution, the IMP/placebo was administered by an unblinded member of the research nursing team.

Both the IMP and placebo were administered by subcutaneous injection using sterile technique. The patient's skin was cleaned with an alcohol swab and allowed to dry prior to administration of the subcutaneous injection. In order to minimise risk of discomfort to the patient the injection was allowed to come to room temperature prior to administration. In addition, the injection site was rotated on a daily basis and the injection was administered slowly (over 30-60seconds) using a 25G needle. The volume of IMP/placebo, date and time of injection and injection site were recorded in the patient notes and in the CRF.

2.11.5 Study drug termination criteria (DFS / RCT)

During the DFS and RCT the study drug was continued until any one of the pre-determined study drug termination criteria was met:

- maximum treatment period (for the RCT this was to be defined following the DFS)
- study drug-related serious adverse reaction (SAR)
- discharge from a critical care environment
- death
- discontinuation of active medical treatment
- the patient, PerLR or ProfLR requests withdrawal from the study
- decision by the attending clinician that the study drug should be discontinued on safety grounds.

In order to minimise the risk of harm to participants related to the potential effect of GM-CSF in expanding the myeloid cell population consideration would be given to to either discontinuing the study drug or reducing the dose by half in the following circumstances:

- WCC > 50,000 cells/mm3
- Platelet count > 500,000 cells/mm3

2.11.6 Drug accountability (DFS / RCT)

On completion of the prescribed dosing schedule for the IMP/placebo all empty, used vials and any unused vials were returned to the Clinical Trials Pharmacy along with the reconstitution log.

During the RCT, vials and reconstitution logs were returned to pharmacy inside a black plastic bag contained within a box by an unblinded member of the research team. Each vial and the black plastic bag was marked with the patient's study number. At the end of the study unallocated and unused study drug was destroyed, with permission from the Sponsor, in accordance with the site pharmacy procedure for the destruction of IMP.

2.12 Blood sample collection (ACS / DFS / RCT)

2.12.1 Healthy volunteers (ACS only)

During the ACS all healthy volunteer blood samples were collected at approximately 8 am on the day of study and always between 8 am and 10 am to match the timing of collection of the critically ill patient blood samples. Each blood sample was taken with the volunteer seated in a reclining chair to minimise the risk of fainting either during or after the procedure. 30mls of blood was collected for participation in the ACS. Following collection of the blood sample the volunteer was asked to rest and given a drink of water or fruit juice along with some biscuits or chocolate. This was in line with procedure followed by the blood transfusion service.

2.12.2 Critically ill patients (ACS / DFS / RCT)

Blood samples from critically ill patients were collected each morning at approximately 8am. A 2-hour time window existed either side of 8 am during which blood samples could be collected depending on the availability of research staff and the logistics surrounding transport of the samples to the research laboratory in Newcastle University. The reason for unifying the timing of collection of the daily blood samples was that there is evidence in both mice and humans to show that diurnal variation in neutrophil function, including phagocytic capacity exists (Shiraishi et al., 1996, Knyszynski et al., 1981). Where possible all blood samples were collected from an indwelling line (arterial line or central venous catheter) to minimise

discomfort to the patient. All blood samples were collected using sterile technique. Following collection blood was placed in a 50ml Falcon tube (Beckton Dickinson Biosciences) for isolation of neutrophils, with 5mls placed in an Ethylenediaminetetraacetic acid (EDTA) tube for flow cytometric analysis and 5 mls placed in a serum gel tube for preparation of autologous serum. The samples were then transported immediately to the laboratory at Newcastle University for analysis. The EDTA tube was stored at 4°C until analysis.

Where no indwelling line was present blood was drawn by venesection from a peripheral vein using aseptic technique.

During both the DFS and the RCT blood samples were collected for safety analysis on a daily basis as part of usual clinical care. These samples were collected by the usual nursing care team and analysed in the hospital laboratory at each site.

2.12.2 Blood sample collection schedule

The table below outlines the blood sample collection schedule for each phase of the study.

Phase of study	D0	D1	D2	D3	D4	D5	D6	D7	D8	D9
ACS	X									
DFS	X	X	X	X	X	X	X	X	X	X
RCT	X		X		X*		X*		X*	

Table 8: Blood sample collection schedule. * During the RCT blood samples were collected on day 4 **or** 5, day 6 **or** 7 and day 8 **or** 9 depending on availability of research laboratory staff.

2.13 Safety, Pharmacovigilance and Trial Oversight (DFS and RCT)

2.13.1 Data Monitoring and Safety Committee

The trial was run in accordance with Good Clinical Practice guidelines. A Data Monitoring and Safety committee was established consisting of 3 independent Intensive Care Clinicians, 1 independent Haematologist and an independent Statistician all of whom had experience of clinical trials. The Data Monitoring and Safety Committee met once prior to the commencement of the DFS. Data were communicated by email during the DFS following the completion of the first and third patient for review by the DMSC prior to the subsequent patients being enrolled. Screening and recruitment were put on hold following the first and third patient until the data had been reviewed and the committee were satisfied that it was safe to proceed with the study. Subsequently the DMSC met via teleconference at 3-6 monthly intervals during the active phases of the DFS and RCT. During the DFS all data was presented in an unblinded fashion. During the RCT blinded and, if requested by the DMSC, unblinded data (prepared by independent statisticians), including adverse events, were presented to the committee at each meeting during open and closed sessions respectively. At each time point the DMSC gave permission for recruitment to continue following review of the available data.

2.13.2 Safety bloods

During the DFS and the RCT safety bloods were collected and analysed for alterations in full blood count (FBC), urea and electrolytes (U&Es) and liver function tests over the 10-day study period. These results were reviewed by the trial study team and appropriate action taken where necessary.

2.13.3 Adverse event reporting

Adverse events were defined as *'any untoward medical occurrence in a patient or clinical trial subject administered a medicinal product and which does not necessarily have a causal relationship with this treatment*.' (Trial protocol Appendix A).

Serious adverse events were defined as '*any adverse event, adverse reaction or unexpected adverse reaction that: a)results in death ; b)is life-threatening (i.e. the*

subject was at risk of death at the time of the event; it does not refer to an event which might have caused death if it were more severe); c)requires hospitalisation or prolongation of existing hospitalisation; d)results in persistent or significant disability or incapacity; e)is a congenital anomaly or birth defect; f)is any other important medical event(s) that carries a real, not hypothetical, risk of one of the above outcomes' (Trial Protocol, Appendix A).

An adverse reaction was defined as any adverse event which was thought to be related to administration of the study drug. Several more common adverse reactions that are recognised to occur with the administration of GM-CSF were recorded on a daily basis in the adverse event (AE) form contained within the e-CRF. These included:

- Skin reaction at the site of injection
- Fever up to 38°C
- Elevated white cell count > 50,000 cells/mm^3
- bone pain
- myalgia
- first dose syndrome

In view of the nature of the study population it was recognised that the likelihood of participants to undergo an adverse event during the study period, as part of their underlying critical illness, was high. Therefore, it was agreed and stated within the trial protocol that any adverse events that were in keeping with the participant's underlying critical illness were not reported as adverse events. All adverse events thought possibly, probably or likely to be related to the study drug were reported and recorded in the adverse event log contained in the CRF.

Because the study was recruiting many patients who had life-threatening conditions it was also expected that patients may suffer serious adverse events related to their underlying critical illness. Therefore, serious adverse events in keeping with the underlying clinical course, and collected as outcomes in the study data e.g. death and organ dysfunction, were not reported as serious adverse events. Any serious adverse events thought possibly, probably or likely to be related to the study drug were recorded as such on the SAE notification forms and faxed to the Newcastle Clinical Trials Unit within 24 hours of the event being recognised. In particular, any

AEs /SAEs occurring within 1 hour of the study drug administration were recommended to be considered as being related. Responsibility for the reporting of AEs lay with the PI at each site. Assessment of causality and expectedness was carried out by the PI or their nominated designated person. Where any doubt existed the PI was required to discuss with the chief investigator who may elect to refer on to the DMSC for further assessment.

The Newcastle Clinical Trials Unit was responsible for onward reporting of any suspected, unexpected serious adverse reactions (SUSARs) to the relevant regulatory authorities and the trial sponsor within the required timeframe. Reporting of all SUSARs to the DMSC in a timely manner was also stipulated.

2.14 Data Collection (all studies)

For the observational Assay Confirmation Study and open-label DFS data were collected on a paper case report form by members of the trial team. CRFs were stored in a locked cupboard within each trial site and underwent monitoring by a member of the Newcastle CTU at regular intervals.

For the RCT an electronic case report form (e-CRF) was developed which was password protected and accessible only to designated members of the trial team. Clinical data collected included patient demographic details, reason for admission to ICU, regular clinical observations, length of stay, selected drug data, SOFA score and incidence of suspected ICUAIs. All clinical data were collected from source data (the ICU observation charts and clinical notes) completed by the usual clinical care team.

2.15 Laboratory Materials

The following laboratory materials were used to carry out laboratory procedures within the three phases of the study. Materials and reagents are listed alphabetically with the supplier and catalogue number in brackets afterwards. Antibodies are listed according to the cellular property of interest with supplier and catalogue number in brackets afterwards.

2.15.1 Plastics

The following plastics were supplied by Beckton Dickinson (BD) Biosciences (Oxford, U.K.): 50ml polypropylene conical Falcon tubes (352070), 15ml polypropylene conical Falcon tube (352096), EDTA coated 3ml blood tubes (367835), Rapid serum gel tubes (368774).

The following plastics were supplied by Greiner Bio-one Limited (Stonehouse, Gloucestershire, U.K.): 50ml polypropylene conical tubes (227261), 15ml polypropylene conical tubes (188271), 5ml serological pipettes (606180), 10ml serological pipettes (607180), 96 well sterile flat bottomed microplates (655101).

The following plastics were supplied by Fisher Scientific (Loughborough, Leicestershire, U.K.): 0.5ml Eppendorf tubes (FB74023), 1.5ml Eppendorf tubes (FB74031), 2ml Eppendorf tubes (FB74111).

The following plastics were supplied by Starlab U.K. Limited (Milton Keynes, U.K.): 3ml graduated Pasteur pipettes (E1414-0311) ,1000µl pipette tips (S1111-2721), 200µl pipette tips (S1111-1700), 0.1-10µl natural pipette tips (S1111-3700).

The following plastics were supplied by Scientific Laboratory Supplies Limited (Nottingham, U.K.): 24-well multiwell plate with lid (sterile) (S3526)

2.15.2 Glassware

The following glassware was supplied by Sigma-Aldrich (Billingham, Dorset, U.K.): Corning microscope slides (CLS294875x25-1440E).

The following glassware was supplied by VWR (Pennsylvania, U.S.) microscope cover slips (631-0150).

2.15.3 Reagents

The following reagents were supplied by Sigma-Aldrich (Billingham, Dorset, U.K.): Agarose 2%, high resolution (A4718), Albumin from bovine serum (A7906), Calcium chloride solution (Ca Cl_2) (21114), Citrate concentrated solution, sodium citrate 3.8% (S5770), Cytochrome C (C2037), Dulbecco's phosphate buffered solution 10x (without Ca^{2+}/Mg^{2+}) (D1408), Dulbecco's phosphate buffered solution 1x (without Ca^{2+}/Mg^{2+}) (D8537), fMLF (N-formyl methinyl leucyl phenylalanine) (47729), Gelatin 2% solution (G1393), Giemsa (48900), Hank's balanced salt solution with Ca^{2+}/Mg^{2+}, HBBS+ (55037), Hank's balanced salt solution without Ca^{2+}/Mg^{2+}, HBSS- (H6648), Methanol (179957), Paraformaldehyde (P6148), Platelet activating factor, PAF (P260), Superoxide dismutase (S5395), Sodium chloride solution (0.9%), NaCl (S5886), Trypan blue (T6146),Tween 20 (P1379), Zymosan A from Saccharomyces cerevisiae (Z4250).

The following reagents were supplied by Pharmacosmos (Holbaek, Denmark): Dextran T500, 6% (5510050090070).

The following reagents were supplied by Invitrogen (Paisley, U.K.): Iscove's modified dulbecco's medium, IMDM (21980065).

The following reagents were supplied by GE Healthcare Lifescience (Little Charlefont, Buckingham, U.K.): Percoll Plus (GZ17544501).

The following reagents were supplied by BD Biosciences (Oxford, U.K.): Pharmlyse (10x concentrate) (555899).

2.15.4 Antibodies

The following antibodies were used to carry out laboratory procedures as documented. Suppliers and catalogue numbers appear in brackets.

2.15.4.1 Neutrophil CD88 expression

PE Rabbit anti-human CD88 (BD Biosciences, 552993)

PE Rabbit IgG isotype control (R&D Systems, ICI05P)

2.15.4.2 Monocyte HLA-DR expression

QuantiBRITE™ PE, Phycoerythrin Fluorescence Quantitation Kit (BD Biosciences, 340495)

QuantiBRITE™ anti-HLA-DR PE, clone L243 / anti-monocyte PerCP-Cy5.5, clone MØP9 (BD Biosciences, 340827)

2.15.4.3 T lymphocyte population studies

Alexa Fluor 488 anti-human CD127 (IL-7Rα), Clone A019D5, isotype mouse IgG1, κ, (Biolegend, 351314)

APC Mouse anti-human CD4, Clone RPA-T4, isotype mouse IgG1, κ, (BD Biosciences 555349)

Brilliant Violet 421 anti-human CD25, Clone M-A251, isotype mouse IgG1,κ, (BD Biosciences, 562442)

PE anti-human CD3, Clone SK7, isotype IgG1, κ, (Biolegend, 344806)

PE-Cy™7 Mouse anti-human CD45RA, Clone HI100, isotype mouse IgG2b, κ, (BD Biosciences 560675)

PerCP anti-human CD45RO, Clone UCHL1, isotype mouse IgG2a (Miltenyi Biotec, 130-095-451)

2.15.4.4 Analysis of Neutrophil Apoptosis

APC Annexin V (Biolegend, 640920)

Propidium iodide solution (Biolegend, 421301)

Annexin V binding buffer solution (Biolegend, 422201)

2.15.4.5 Analysis of serum GM-CSF concentration

Human GM-CSF ELISA kit (BD Biosciences, 555126)

2.16 Laboratory Procedures

2.16.1 Isolation of neutrophils from whole blood (ACS / DFS / RCT)

Neutrophils were isolated from whole blood by dextran sedimentation and percoll gradient separation. Samples were collected in a 50 ml Falcon tube containing 1ml sodium citrate 4% /10mls blood. When samples arrived in the laboratory whole blood was centrifuged at 300g with no brake for 20mins at room temperature.

Following centrifugation the plasma was transferred to a glass vial and placed in a water bath at 37°C with calcium chloride ($CaCl_2$, 220µl per 10ml plasma). Warmed, filtered dextran was added to the remaining cell pellet at a volume of 2.5mls per 10mls of cell pellet. This suspension was then made up to the original blood volume with warmed 0.9% saline. The Falcon tube was inverted gently to ensure the suspension was fully mixed. The lid was loosened and the suspension left to stand at room temperature for 30 minutes to allow sedimentation.

After 30 minutes the leucocyte rich upper layer was aspirated and transferred to a new Falcon tube where it was made up to 50mls with warmed saline and centrifuged at 200g for 5 minutes at room temperature.

Following centrifugation, the supernatant was discarded and the cell pellet was re-suspended in 2.5mls of 55% Percoll and layered on a 70%/81% Percoll gradient. The gradient was centrifuged at 700g with zero brake for 20 minutes at room temperature, to allow separation of the cells.

After centrifugation the neutrophils were collected from the 70/81% interface and transferred to a new Falcon tube (Figure 2d). The cells were then washed in Hanks Balanced Salt Solution without calcium and magnesium (HBSS-) and centrifuged at 200g with zero brake for 5 minutes at room temperature.

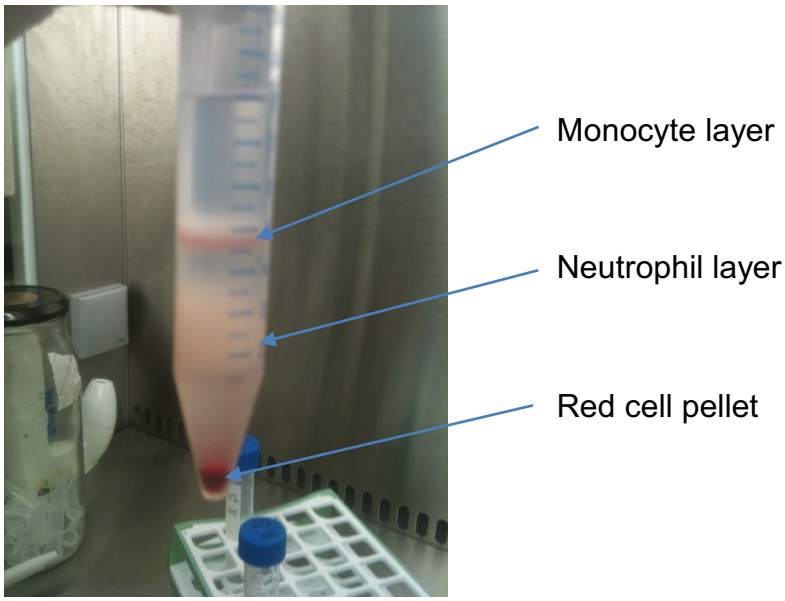

Figure 2d: Cell populations within percoll gradient ready for harvesting

2.16.2 Cell count (ACS / DFS / RCT)

Following isolation 100µl of suspended neutrophils were mixed with 5µl of trypan blue and placed in the chamber of a haemocytometer to perform a cell count using light microscopy. Using the known dilution factor the total number of isolated neutrophils was calculated for each sample.

2.16.3 Assessment of neutrophil phagocytic capacity (ACS / DFS / RCT)

The cells were re-washed (200g for 5 minutes, room temperature) and re-suspended in Iscove's Modified Dulbecco's Medium (IMDM) at a concentration of 1million cells/ml. 500ul of neutrophil suspension was pipetted into 4 wells of a 24 well plate.

The cells were incubated for 30 minutes with 1% autologous serum at 37°C in a 5% CO_2 incubator. Zymosan was opsonised in autologous serum at 37°C in a water bath for 30 minutes. After opsonisation the zymosan was washed twice in IMDM at 10,000g for 1 minute at room temperature and re-suspended in 100µl of IMDM. 0.02mg of zymosan was added to the neutrophils in 3 of the 4 wells. The plate was then incubated for a further 30 minutes at 37°C in a 5% CO_2. After incubation was completed the wells were washed 3 times with Phosphate Buffered Saline (PBS), fixed with methanol (200µl per well) and stained with Giemsa 1:10 for 15 minutes. Finally, the cells were washed with deionised water until aspirating clear and allowed to air dry.

Using light microscopy, the number of neutrophils containing 2 or more zymosan particles were then counted and expressed as a percentage of the total number of neutrophils assessed. Four fields of 100 cells / field were counted in each well.

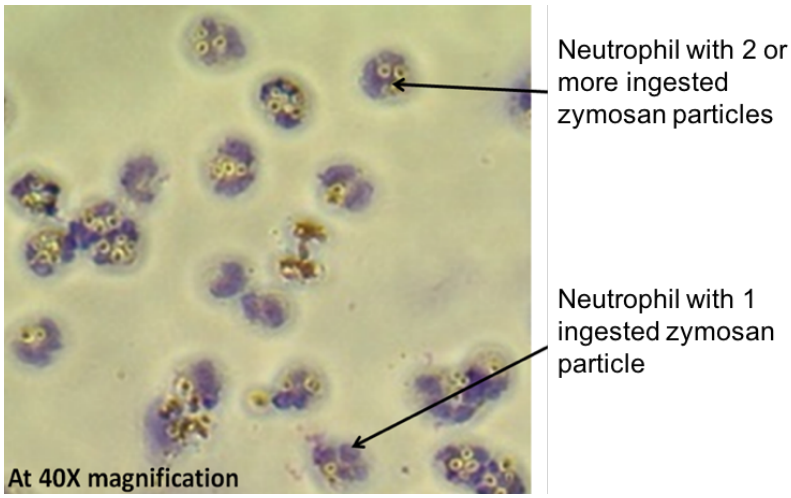

Figure 2e: Assessing neutrophil phagocytic capacity by light microscopy. the number of neutrophils containing 2 or more zymosan particles were then counted (four fields of 100 cells per well) and expressed as a percentage of the total number of neutrophils assessed.

2.16.4 Assessment of neutrophil CD88 expression (ACS/ DFS / RCT)

Flow cytometry was used to assess neutrophil CD88 expression in all patients.

Whole blood was collected in EDTA as described and stored at 4°C until analysis. 50ul of blood was placed in each of three 1.5ml Eppendorf tubes (a, b and c). The blood was then incubated unstained (a), with 30µl PE rabbit IgG isotype control (b) or with 15µl PE Rabbit anti-human CD88 (c) for 30mins at 4°C in the dark. Following incubation 1.4ml of 1x Pharmlyse solution (10x Pharmlyse solution was diluted with de-ionised water pH 7.3) was added to each Eppendorf tube, which was then vortexed, and placed in the dark for 20 minutes at room temperature to allow red cell lysis to take place. Following lysis, the samples were transferred to labelled FACS tubes and washed 3 times in a FACS wash machine before being analysed. Analysis was performed by flow cytometry on FACS Canto. Neutrophils were identified by forward scatter and side scatter and gated accordingly. Mean, median and geometric mean fluorescence were recorded for CD88.

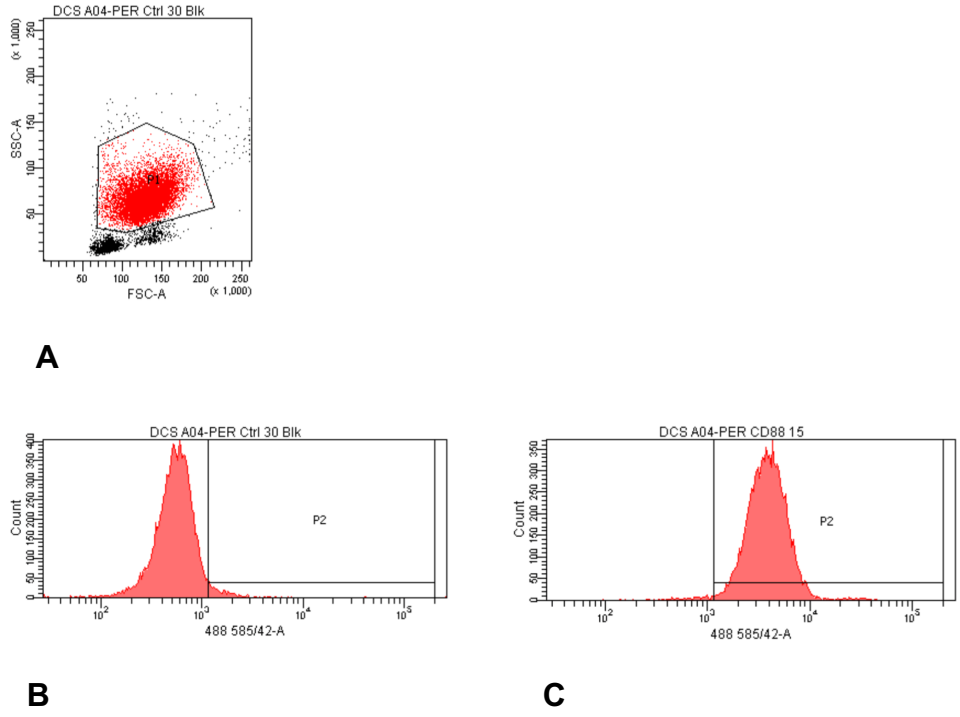

Figure 2f: Assessing neutrophil surface CD88 expression by flow cytometry.
A) Neutrophils were identified and distinguished from monocytes and lymphocytes by forward scatter (FSC-A) and side scatter (SSC-A) characteristics and gated accordingly (P1). Neutrophil CD88 PE expression isotype control sample (B) and stained neutrophils (C). Example flow scatter plot shown from subject A04.

2.16.5 Assessment of monocyte HLA-DR expression (ACS / DFS / RCT)

Monocyte HLA-DR expression (antibodies bound per cell (Ab/cell) was calculated by flow cytometry using QuantiBRITE^{TM} Anti-HLA-DR PE/ Anti-monocyte PerCP-Cy5.5 QuantiBRITE^{TM} PE Fluoresecence Quantitation Kit (BD Biosciences).

Prior to sampling fluorescence quantitation was performed using the QuantiBRITE^{TM} Phycoerythrin Fluorescence Quantitation Kit. Kits were stored at 4°C. The lyophilised pellet of beads conjugated with four levels of lot-specific PE was reconstituted with 500μl flow buffer (PBS + 0.2% BSA) and vortexed. The tube was run on a FACS Canto machine and bead singlets identified and gated using forward scatter and side scatter. 10,000 events were collected. The 4 bead peaks were selected on a histogram plot and the geometric means displayed. The lot-specific values for PE molecules per bead were plotted against the recorded GMFs to calculate the correlation co-efficient to be applied to calculate a sample's ABC.

Whole blood was collected in EDTA as described and kept at 4°C until analysis commenced. 50μl of blood was placed into a 1.5ml Eppendorf tube and 20μl of QuantiBRITE^{TM} PE solution added. The blood was then vortexed. The test Eppendorf and matched control were placed in the dark at room temperature for 30mins until incubation was complete. Following incubation cells were lysed with 1.4ml Pharmlyse solution in the dark for 20 minutes. Cells were then transferred to FACS tubes and washed 3 times in the FACS wash machine prior to analysis.

Monocytes were identified initially by side scatter and forward scatter characteristics and then by PerCP-Cy5.5 (CD14 / CD64) positivity to ensure detection of all monocytes (both CD14 high and CD14 low). Once the monocyte population had been identified the HLA-DR geometric mean fluorescence was recorded and the antibodies per cell calculated using the correlation co-efficient determined during the quantitation analysis. Analysis was performed by flow cytometry on FACS Canto.

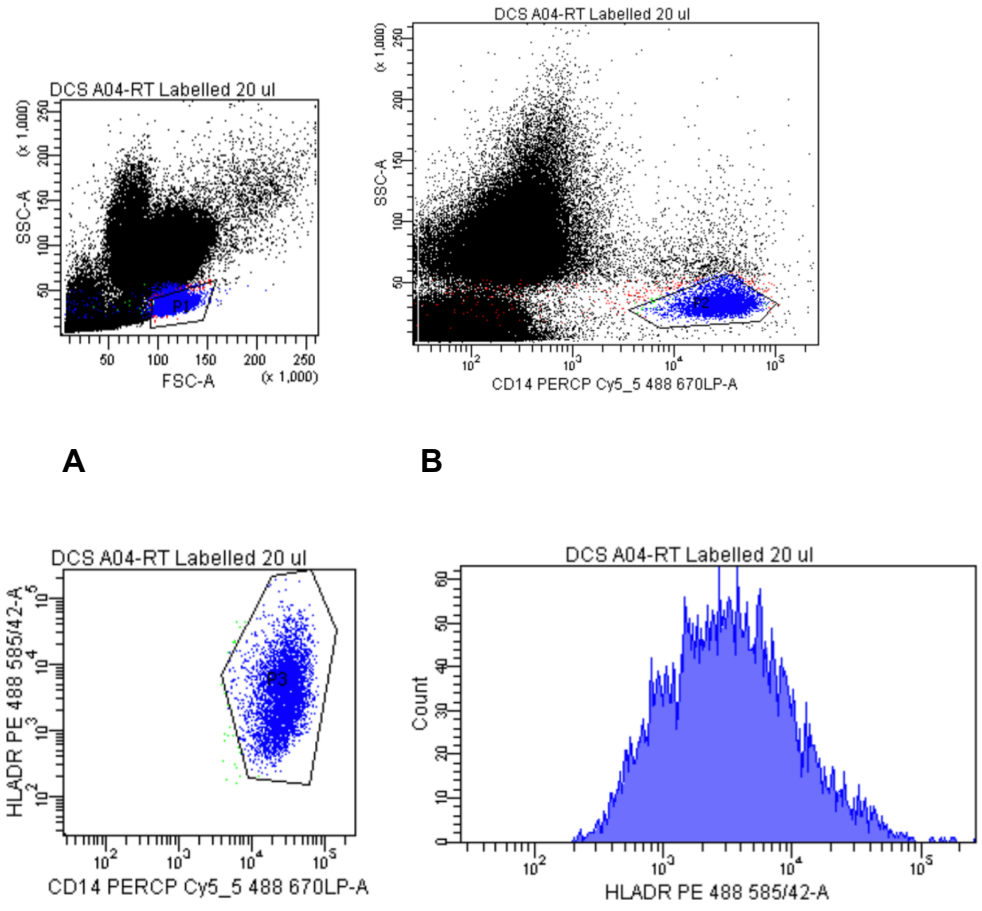

Figure 2g: Assessment of monocyte HLA-DR expression by flow cytometry. Monocytes were gated by forward scatter, side scatter and CD14+CD64+ characteristics and gated accordingly (A+B). mHLA-DR expression was assessed by measurement of geometric mean fluorescence and the number of antibodies per cell calculated using the correlation co-efficient determined during the quantitation analysis.

2.16.6 Measurement of superoxide anion release by neutrophils (RCT only)

Measurement of superoxide release by neutrophils was determined by the calculating the amount of superoxide dismutase-inhibitable reduction of cytochrome C. Isolated neutrophils were re-suspended in HBSS containing calcium and magnesium (HBSS+) at a concentration of 10 million cells /ml. 50µl of the cell suspension (500,000 cells) were placed in each of 8 labelled Eppendorf tubes as outlined in table 9.

Cells were primed (P) with 0.5µl PAF (100nM) or unprimed (H) and incubated with an equivalent volume of HBSS+. Incubation took place in a shaking water bath at 37°C for 10 minutes.

Following priming cells were either stimulated (F) with formyl methionine leucine phenylalanine fMLF (50µl, 100nM end concentration) or made up to the equivalent volume with HBSS+ control (50µl). 25µl of superoxide dismutase (S) was added to four of the Eppendorf tubes with an equivalent volume of HBSS+ being added to the other four tubes as a control. Finally, 375µl of cytochrome C (1mg/ml end concentration) was added to each of the eight Eppendorf tubes. The cells were again incubated in a shaking water bath for 10-15 minutes at 37°C.

Eppendorf	Priming agent	Stimulation	Superoxide dismutase
H	None	None	No
HS	None	None	Yes
HF	None	fMLF	No
HFS	None	fMLF	Yes
P	PAF	None	No
PS	PAF	None	Yes
PF	PAF	fMLF	No
PFS	PAF	fMLF	Yes

Table 9: Measurement of superoxide anion release in neutrophils. PAF, platelet activating factor; fMLF, formyl methionine leucine phenylalanine.

Following incubation, the reaction was stopped by placing the cells on ice for 5 minutes. The cell suspensions were then centrifuged at 10,000g for 3 minutes at 4°C and the supernatants transferred into a 96 well plate (100µl/well). The plate was read as soon as possible at 550nm using a plate reader. The generation of O_2^- was determined by the amount of superoxide dismutase-inhibitable reduction of cytochrome c which occurred. Results were expressed as nanomoles of superoxide generated per 10^6 neutrophils (nmol/10^6 neuts).

2.16.7 Assessment of neutrophil chemotaxis (RCT only)

Neutrophil chemotaxis was assessed by the under agarose method (Nelson et al., 1975). In advance glass microscope slides were cleaned with ethanol and dried with lens cleaning tissue. The slides were dipped in gelatine solution and allowed to dry. Agarose medium was prepared by combining 2ml of 25% Bovine Serum Albumin (BSA) with 6.25ml of 2% gelatine and 25ml of 2% agarose. The medium was made up to 50 ml with IMDM. Using a pipette, 5ml of the agarose medium was slowly applied to the surface of each coated slide ensuring that no bubbles appeared within the medium. The slides were left to set overnight. Once prepared the slides were stored at 4°C and used within 48 hours.

Immediately prior to use a template was used to cut 3 wells into each side of the slide. The central plug of agarose was gently aspirated from each well using a vacuum pump taking care not to damage the integrity of the well.

Freshly isolated neutrophils were re-suspended at 25x10^6/ml in IMDM containing 1% autologous serum.

10µl of the neutrophil suspension (250,000 cells) were placed in each central well. The chemoattractant (fMLF100nM) was placed in one adjacent well and IMDM alone (as a control) in the other adjacent well. This was duplicated on each slide.

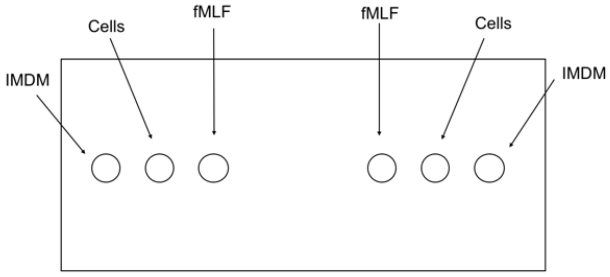

Figure 2h: Assessment of neutrophil chemotaxis by under agarose method.

The slides were incubated at 37°C in a 5% CO_2 incubator for 2 hours. Following incubation, the slides were fixed in 2.5% paraformaldehyde overnight before being rinsed in deionised water, allowed to air dry and stained with Giemsa solution.

The chemotaxis migration distance (μm) was measured using computer image analysis. The mean neutrophil chemotaxis migration distance was measured by subtracting the distance between the origin of the cells and the leading edge of cells migrating towards the IMDM control (i.e. random migration) from the distance between the origin of the cells and the leading edge of the cells migrating towards the fMLF chemoattractant.

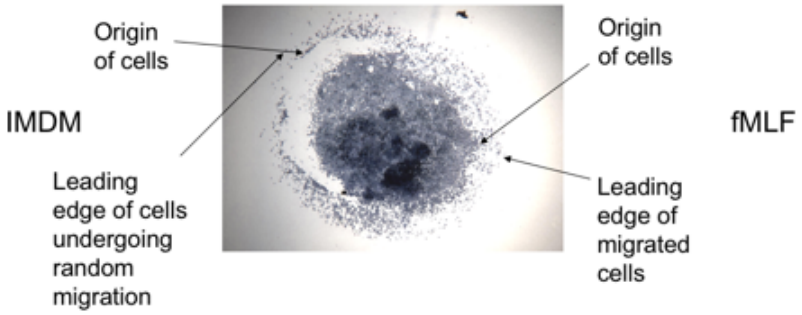

Figure 2i: Assessment of neutrophil migration distance by computer imaging analysis.

2.16.8 Assessment of neutrophil apoptosis (RCT only)

Neutrophil apoptosis was assessed by flow cytometry.

Isolated neutrophils were re-suspended in HBSS- at $10x10^6$/ml. 100µl of cells (1 million cells) were placed into each of 2 Eppendorf tubes (one for control(a) and one for stained cells(b)). Both Eppendorf tubes were centrifuged at 300g for 3 minutes at room temperature. The supernatants were removed and each of the cell pellets was re-suspended in 100µl of Annexin V buffer (pre-diluted 1:10). 5µl of Annexin V allophycocyanin (APC) was added to the test sample (b). The Eppendorf tubes were incubated in the dark at room temperature for 10 minutes. After 10 minutes 1µl of propidium iodide (PI) was added to the test sample (b). Both Eppendorf tubes were topped up with 200µl Annexin V buffer and incubated for a further 10 minutes in the dark. The cells were then manually washed and re-suspended in 200µl Annexin V buffer before being analysed.

The samples were analysed by flow cytometry on a FACS Canto machine. Early apoptosis was identified in those cells staining positive for Annexin V but negative for PI. Late apoptosis was identified in those cells staining positive for both Annexin V and PI.

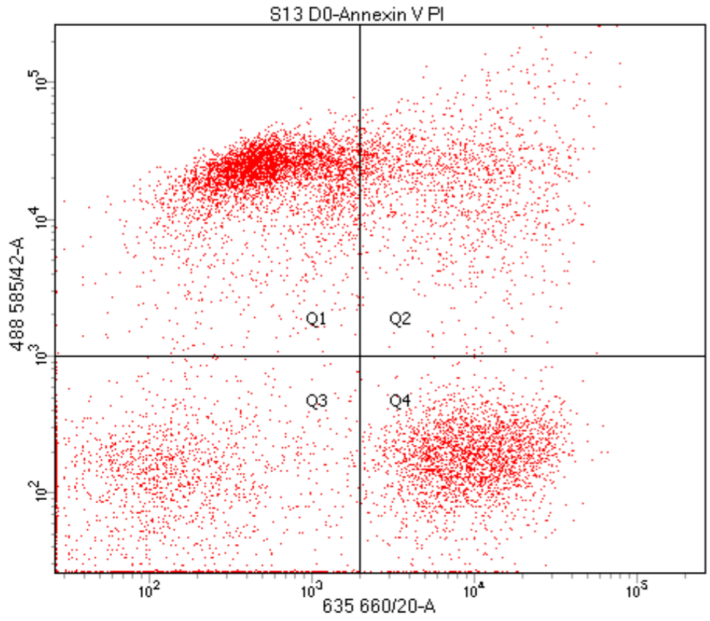

Figure 2j: Assessment of neutrophil apoptosis by flow cytometry. Early apoptosis was identified in cells staining positively for Annexin V APC (635 660/20-A) and negatively for propidium iodide (488 585/42-A) – Q4. While late apoptosis was identified in cells staining positively for both Annexin V APC (635 660/20-A) and propidium iodide (488 585/42-A) – Q2

2.16.9 Assessment of regulatory T cell proportions (RCT only)

The regulatory T cell population was identified by flow cytometry. Whole blood was collected in EDTA as described and kept at 4°C until analysis commenced. 50ul of blood was placed into four 1.5ml Eppendorf tubes and a panel of antibodies was applied to enable identification of the total T lymphocyte population and the subset of regulatory T cells (Tregs) including differentiation of the memory and naïve fractions of the Treg population. An unstained sample acted as a control for these studies. Antibody staining took place as described in Table10.

Cells of interest	Antibodies
T lymphocytes	CD3, CD4, CD25, CD127, CD45RO, CD45RA
Memory Tregs	T lymphocyte panel minus CD45 RA
Naïve Tregs	T lymphocyte panel minus CD45 RO

Table 10: Fluorochrome labelled antibodies used for assessment of regulatory T cell proportions. Treg, regulatory T cells.

Following addition of the antibodies the Eppendorf tubes were incubated at 4°C in the dark for 30 minutes. On completion of the incubation period 1.4mls of Pharmlyse was added to each Eppendorf. The Eppendorfs were then vortexed, and placed in the dark for 20 minutes at room temperature to allow red cell lysis to take place. Following lysis, the samples were transferred to labelled FACS tubes and washed 3 times in a FACS wash machine before being analysed. CD4+ T lymphocytes were identified by gating on CD3 and CD4 positive cells. Regulatory T cells were identified as those which were CD25+ and CD127 low within the T cell population. The percentage of Tregs as a proportion of the total T cell population was calculated. Memory T cells were identified as those which were $CD3^{+}CD4^{+}CD25^{+}CD127_{low}$ $CD45RO^{+}CD45RA^{-}$ while naïve T cells were $CD3^{+}CD4^{+}CD25^{+}CD127_{low}$ $CD45RA^{+}CD45RO^{-}$.

2.16.10 Serum measurement of GM-CSF concentration (DFS only)

Serum GM-CSF concentration was measured by ELISA using a Human GM-CSF ELISA SET (BD Biosciences).

The standard used was recombinant human GM-CSF. During preparation of the standard 148ng of the lyophilised product was diluted in 4.93ml of sterile water giving a final concentration of 30ng/ml. A 6µl aliquot of this solution was then further diluted into 594µl giving a stock standard at a concentration of 300pg/ml. Serial dilution took place to give 7 standards (300pg/ml, 150pg/ml, 75pg/ml, 37.5pg/ml, 18.8pg/ml, 9.4pg/ml, 4.7pg/ml).

The required number of microwells on a 96 well plate were coated with 50µl of capture antibody (anti-human GM-CSF diluted 1:250 with coating buffer (0.1M bicarbonate buffer, pH 9.5)), covered with parafilm and placed in the fridge overnight. The following morning excess coating antibody/buffer solution was thrown away and the wells were covered with 200µl of assay buffer (PBS containing 0.5% BSA and 0.1% tween). The plates were incubated at room temperature for 1 hour. During the incubation dilutions of the participant serum samples took place. After 1 hour the blocking solution was aspirated and the plates were washed 3 times with PBS tween and blotted on absorbent paper. 100µl of each standard, serum sample and control were applied to the relevant microwells on the 96 well plate. The plate was sealed and incubated at room temperature for 2 hours. Following incubation each well of the plate was aspirated and washed with PBS Tween 5 times. After the final wash the plate was inverted and blotted on absorbent paper to remove any residual solution form the wells. 100µl of working detector (containing detection antibody; biotinylated anti-human GM-CSF and the enzyme reagent; streptavidin-horseradish peroxidase conjugate, diluted 1:250 in assay buffer) was added to each well. The plate was once again sealed and incubated for 1 hour at room temperature. Following incubation, the plate was aspirated and washed seven times and100µl of substrate solution was added to each well. The plate was incubated unsealed for 30 minutes at room temperature in the dark. Finally, 50µl of stop solution was added to each well and the plate was transferred to the plate reader. Following labelling of standards absorbance was read at 450nm within 30 minutes of the reaction stopping.

2.16.11 Storage of samples (ACS / DFS / RCT)

All samples of whole blood for collection of serum or plasma were centrifuged as soon as possible after collection. Plasma / serum was aspirated carefully using a Pasteur pipette and placed into 1.5ml Eppendorf tubes in aliquots of 750μl. The Eppendorf tubes were labelled with the patient study number, the date and corresponding study day on which the sample was collected, and the nature of the sample. The samples were then stored in a locked freezer at -80°C until required for analysis. Following completion of the trial the samples will be stored for up to 15 years. Consent for storage of all samples was taken at the time of enrolment into the study.

Flow cytometry samples were discarded after analysis had been completed on the day of collection.

Cytospin slides of isolated neutrophils were labelled with the study identification number and date and corresponding study day of sample and will be stored in the chief investigators laboratory for up to 15 years.

2.17 Clinical Assessments /Data Scores

2.17.1 Sequential Organ Failure Assessment Score

The Sequential Organ Failure Assessment Score (SOFA score) was used to measure patient organ dysfunction / morbidity over the course of the study. The SOFA score is an internationally recognised tool for assessing acute organ dysfunction and failure in critically ill patients (Vincent et el., 1998). It is composed of 6 parameters assessing respiratory, renal, hepatic, cardiovascular, haematological and neurological organ function. It is an objective, useful tool for assessing change in status of organ function over time and has been widely used in clinical trials for this purpose.

Organ system	**0**	**1**	**2**	**3**	**4**
Respiratory PaO2/FiO2 (mmHg)	> 400	≤ 400	≤ 300	≤ 200	≤ 100
Renal Creatinine (µmol/L)	< 106	106-168	169-300	300-433 urine < 500ml/day	>433 urine < 200ml/day
Hepatic Bilirubin (µmol/L)	<20	20-32	33-100	101-203	>203
Cardiovascular Hypotension	No hypotension	MAP < 70mmhg	Dopamine ≤5µg/kg/min	Dopamine <5 or Epinephrine ≤0.1 or Norepinephrine ≤ 0.1 µg/kg/min	Dopamine >15 or Epinephrine ≥ 0.1 or Norepinephrine ≥ 0.1 µg/kg/min
Haematological Platelet count ($x10^3/mm^3$)	>150	≤ 150	≤ 100	≤ 50	≤ 20
Neurological GCS	15	13-14	10-12	6-9	< 6

Table 11: Sequential organ failure assessment score (SOFA Score). MAP, mean arterial pressure; PaO2, partial pressure of oxygen; Fio2, inspired oxygen concentration; GCS, Glasgow Coma Scale

2.17.2 Acute Physiology and Chronic Health Evaluation Score

The Acute Physiology and Chronic Health Evaluation (APACHE) II Score was introduced in 1981 as a modification of the original APACHE score. It is widely used across the world to evaluate the severity of disease in patients admitted to intensive care environments (Knaus et al., 1985). The score takes into account the patient's age, the severity of their acute illness and their co-morbid disease status. It has been validated as a tool with which to predict mortality from critical illness. The score is calculated within the first 24 hours of admission to intensive care. The score was recorded for all patients entering the trial.

2.17.3 Incidence of ICU-Acquired Infection

Incidence of ICUAI was determined using the Hospitals in Europe Link for Infection Control through Surveillance (HELICS) criteria. The HELICS criteria were devised as part of a European project to standardise methods of surveillance for hospital-acquired infection (HAI) and enable comparison of infection rates across international, national and regional healthcare networks (Mertens et al.,1996). As well as identifying rates of infection the framework enables identification of factors which may help prevent the acquisition of HAI (HELICS Protocol 6.1, 2004).)

During the RCT data were collected enabling assessment of the acquisition of ICUAI according to the HELICS criteria. These data included; clinical suspicion of infection, temperature, heart rate, white cell count, microbiological samples requested and corresponding results, use of indwelling catheters and use of antibiotic therapy. All patients were assessed for the development of ICUAI up to day 30.

2.17.4 PaO_2:FiO_2

The PaO2:FiO2 is calculated by dividing the partial pressure of oxygen (PaO2) in kilopascals by the inspired oxygen fraction as a fraction of 1 (e.g. breathing room air, 21% O2 equals an inspired oxygen concentration of 0.21). The partial pressure of oxygen was measured by arterial blood gas analysis at least once a day as part of usual clinical care with blood samples drawn from an indwelling arterial line. The inspired oxygen fraction was recorded on the ICU observation chart at hourly intervals

2.18 Statistical analysis

2.18.1 Assay confirmation study

The assay confirmation was designed solely to examine the correlation between neutrophil CD88 expression and neutrophil phagocytic capacity when analysed within our laboratory. Minimal demographic data was collected and no comparative analysis was performed. Descriptive statistics were used to describe the results. The correlation between CD88 expression and phagocytic capacity was examined by Spearman's correlation.

2.18.2 Dose finding study

The sample size for the DFS was derived pragmatically. A sample size of n=6 per group (24 in total) was calculated to provide 80% power to detect an effect size of 1.8 at a significance level of 0.05 between any 2 groups using the 2-sample t-test.

Data for the primary and secondary outcomes will be summarized within each dosing group using descriptive statistics (means, medians, standard deviations, interquartile-ranges for continuous data and frequencies, proportions and rates for dichotomous and categorical data) with confidence intervals. Descriptive summaries of demographic and operational data will be presented in a similar way. No comparative hypothesis testing or statistical modelling will be carried out.

Safety data will also be reported in a descriptive fashion. Missing data will be described but no data replacement or imputation will be carried out.

Analysis will be carried out using GraphPad Prism computer software.

If low dose, short duration (3μg/kg/day) GM-CSF is demonstrated to be

effective (i.e. we observe a phagocytosis rate on day 2 that is protective against infection (≥50% in all patients) and if there is no toxicity observed, then we will proceed directly to RCT at that dose. Higher doses would then seem unlikely to further reduce infection risk significantly, while carrying an increased risk of toxicity.

2.18.3 Randomised controlled trial

For the RCT, on the basis of the group's prior ex-vivo data in critically ill patients, showing a mean rate of neutrophil phagocytosis of 39% (standard deviation (SD) 13%) (Conway Morris et al 2009), we believed an effect size incorporating an absolute increase of at least 15% (i.e. from 39% to 54% mean phagocytosis) would be clinically important. Our power calculation estimated that a sample size of 17 in each group would give a power of 90% to detect a difference (absolute difference in mean phagocytosis of 15%) between the groups at 2 days with a significance level of 0.05 using the 2-sample t-test. This was equivalent to an effect size of 1.15 with respect to change from baseline within the two groups. To allow for an attrition rate of approximately 10% we planned to recruit 38 patients (19 per group).

The statistical analysis plan for the RCT was devised by the independent statisticians (Dr Thomas Chadwick and Dr Anthony Fouweather, Department of Statistics, Institute of Health and Society, Newcastle University) and agreed by the PMG and DMSC prior to any analysis being undertaken.

As this study was a small phase 2 clinical study, the analysis planned outlined that descriptive statistics would principally be reported including means and standard deviation for continuous data, frequency, median and inter-quartile range for ordinal data and proportions / rates for dichotomous / categorical data. Confidence intervals would be reported as appropriate. Baseline demographic and clinical data (including the SOFA score) would be described and summarised by group.

Adverse events, serious adverse events and suspected unexpected serious adverse events would be summarised by group but would not be undergo any formal statistical analysis. Operational data including withdrawals etc. would be reported in a similar manner.

The primary outcome (neutrophil phagocytic capacity on day 2 following administration of GM-CSF) would undergo comparative analysis, between the two treatment arms, by two-sample t-test however this would be exploratory rather than definitive in nature due to the small sample size. Where the data was considered to be non-normal the Mann Whitney U test for non-parametric analysis would be used.

Where appropriate Analysis of Covariance (ANCOVA) would be used to adjust for differences between the groups in relation to baseline values or site effect.

Secondary outcomes including laboratory and some clinical data would be examined using similar methods including comparative analysis by two-sample t-test or Mann Whitney U test as appropriate. Dichotomous variables would be analysed by Fisher's exact test.

Data would initially be analysed on an intention to treat basis. Following this per-protocol analysis (including patients receiving at least 2 doses of GM-CSF / placebo) would be carried out.

Secondary clinical outcomes including length of stay on ICU and in hospital, duration of mechanical ventilation and incidence of ICUAIs would be summarised by group.

Statistical significance was set at 0.05.

Descriptive statistics would be used for both the incidence of ICUAIs and 30-day mortality as the study was not adequately powered to assess these. Laboratory safety measures would be subject to comparative analysis as described for the secondary outcome measures above.

Missing data (other than due to mortality) would be described and examined to determine the extent of and reason for such omissions. Multiple imputation techniques may be considered for the primary outcome but given the exploratory nature of this study the value of such data may be limited.

For the purposes of this thesis, I conducted my own independent statistical analysis following the same analysis plan as had been set out by the independent study statisticians. I previously described I used GaphPad Prism statistical analysis software to conduct all analyses. I assessed normality of data for each data set using the D'Agostino-Pearson test (as recommended by the GraphPad Prism software) and used the outcome of this to determine the appropriate statistical test; using the 2-sample t-test for normally distributed, parametric data and the Mann Whitney U test for non-normal, non-parametric data.

Chapter 3: Assay Confirmation Study

This chapter will outline the results of the preliminary phase of the study, the assay confirmation study. It will begin by stating the aim of the study, then describe the screening, recruitment and consent process, and subsequently go on to report the results. Lessons learnt during screening and recruitment and the impact of the results on the subsequent phases of the study will then be discussed along with the limitations and conclusions of the ACS.

3.1 Aim of study

The assay confirmation study (ACS) was designed to determine whether neutrophil surface CD88 expression could reliably be used as a surrogate marker for neutrophil phagocytic capacity in identifying patients eligible for participation in the DFS/RCT. As outlined previously background work leading to this study had identified a significant correlation between neutrophil surface CD88 (C5aR) expression and neutrophil phagocytic capacity (Conway Morris et al., 2009, Conway Morris et al., 2011). In the search for rapid diagnostics and biomarker-guided therapies we tested whether neutrophil CD88 could be used as a biomarker in this setting.

3.2 Recruitment

The ACS ran from August 2012 to December 2012 inclusive. All adult admissions to 3 intensive care units in the North East of England (Royal Victoria Infirmary General ICU, Freeman Hospital Critical Care Unit and Queen Elizabeth Hospital, Gateshead Critical Care Unit) were screened for eligibility to take part in this phase of the study using the same inclusion and exclusion criteria as would be used in the subsequent phases of the study (see Table 7, Chapter 2; section 2.7). All patients were screened each morning for up to 48 hours following their admission. Screening did not take place at weekends when no researcher was available. More than a thousand screening episodes took place on more than 500 patients from which a total of 16 patients were found to be eligible to participate. Consent to participate in the study was provided by the patient in three cases, a personal legal representative in 10 cases and a professional legal representative in 2 cases. One patient declined to

give consent and therefore a total of 15 patients were recruited. No patients withdrew, or were withdrawn, from the study following consent. The commonest reasons for exclusion from the study were abnormalities in full blood count parameters (haemoglobin <8.5g/dL, total white cell count >20 x 10^9/L, platelet count < 50 x 10^9/L), a history of cancer and the use of maintenance immunosuppressive drugs. Following acquisition of consent, a single 20mL blood sample was collected from each patient for assessment of CD88 expression, neutrophil phagocytic capacity and HLA-DR expression. All blood samples were collected at approximately the same time in the morning (median time 08:10 am, range 07:55 – 10:10 am) and were processed as soon as possible after collection (within 1 hour).

3.3 Demographic Data

Mean age was 64.5 years (s.d +/- 15.5, range 31-85). Male to female ratio was 7:8. There was a wide range of diagnoses responsible for the underlying critical illness with 8 patients having been admitted with a primary surgical diagnosis and 7 a primary medical diagnosis.

In the healthy volunteer population mean age was 38.1 years (s.d +/- 7.5, range 30 – 43). Male to female ration was 4:5.

The table below summarises the demographic and diagnostic data for the critically ill patients included in the ACS.

Patient Study No	Age (years)	Sex	Study Site	Reason for admission
A01	78	F	RVI	Oesophageal perforation
A02	85	M	RVI	Gallbladder empyema
A03	78	M	FRH	Femoral embolectomy / fasciotomy
A04	61	M	FRH	Liver failure, GI bleed / cirrhosis
A05	80	M	RVI	Community acquired pneumonia
A06	78	F	FRH	Pancreatitis
A07	62	M	FRH	Elective AAA with post-op resp failure
A08	31	F	FRH	Gallstone pancreatitis
A09	66	M	QEH	ALD, sepsis, renal failure
A10	52	F	RVI	Cellulitis, renal failure
A11	71	F	QEH	Rhabdomyolysis
A12	38	M	FRH	Pancreatitis
A13	68	F	QEH	Bowel obstruction
A14	65	F	RVI	Exacerbation of COPD / CAP
A15	55	F	QEH	Asthma exacerbation

Table 12 Demographic data for ACS participants

AAA, abdominal aortic aneurysm; ALD, alcoholic liver disease; CAP, community-acquired pneumonia; COPD, chronic obstructive pulmonary disease; FRH, Freeman Hospital, Newcastle upon Tyne; GI, gastrointestinal; QEH, Queen Elizabeth Hospital, Gateshead; RVI, Royal Victoria Infirmary, Newcastle upon Tyne.

3.4 Neutrophil phagocytic capacity

3.4.1 Critically ill patients

Neutrophils were extracted from whole blood and isolated by dextran sedimentation and a percoll gradient separation technique as outlined in chapter 2 (section 2.16.1). Phagocytic capacity was then assessed by light microscopy following incubation of neutrophils with zymosan that had been opsonised in autologous serum.

In one patient's sample (DCS-A01) the neutrophil extraction process failed due to loss of cells from the 24 well plate during the final wash. This was thought to be due to contamination of the PBS used in the washing process. As a result, the phagocytic capacity could not be measured in this patient. Results were available for the remaining 14 patients. Neutrophil phagocytosis (defined as the proportion of neutrophils ingesting ≥2 zymosan particles) ranged from 7.3% to 53.7%. These values fell within the expected range for a population of critically ill patients. Overall mean neutrophil phagocytosis was 34.8% (s.d +/- 13). Only one patient (1/14, 7.1%) had neutrophil phagocytic capacity greater than or equal to 50% (i.e. adequate phagocytic capacity).

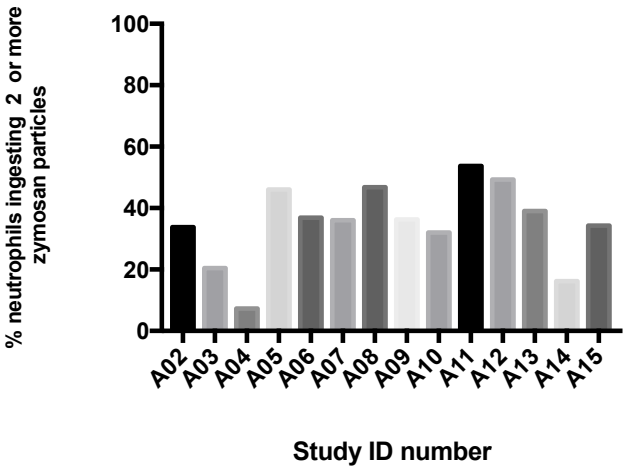

Figure 3a: Individual mean phagocytic capacity in critically ill patients
Phagocytosis assay failed for 1 patient, n=14.

3.4.2 Healthy Volunteers

Healthy volunteer blood samples from university staff and students were analysed simultaneously. Due to the cohort of donors these samples were not matched for age and sex. As with the patient samples these samples were collected around 8 am and processed as soon as possible and always within 1 hour from the time of collection. The samples showed a similar phagocytic rate compared with previously studied healthy cohorts with phagocytic capacity ranging from 44.8 -90% (Conway Morris et al., 2009). Mean phagocytic capacity was 74% (s.d. +/- 14.8).

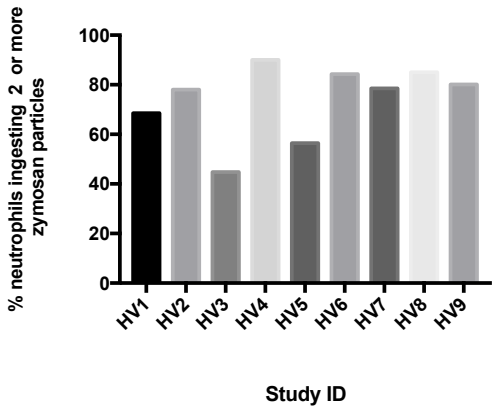

Figure 3b: Individual mean phagocytic capacity in healthy volunteers (HV).

3.4.3 Comparison between critically ill patients and healthy volunteers

There was a statistically significant difference in mean neutrophil phagocytic capacity between the two groups when analysed by two-sample t-test.

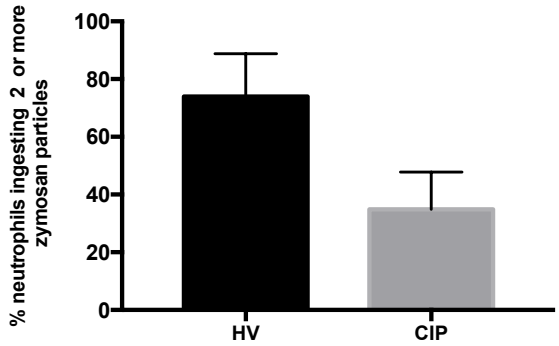

Figure 3c: Mean phagocytic capacity healthy volunteers and critically ill patients. Healthy volunteers (HV) mean phagocytic capacity 74% (s.d. +/-14.8%) compared with critically ill patients (CIP) 34.8% (s.d. +/- 13%) P <0.001 by two-sample t-test.

3.5 Assessment of CD88 (C5aR) Expression

As previously described (chapter 2, section 2.16.4) blood was collected in an EDTA tube for analysis of neutrophil surface CD88 expression by flow cytometry. Whole blood was incubated at 4°C with CD88 PE stain prior to red cell lysis by pharmlyse. Finally, the cells were washed prior to analysis.

CD88 expression was measured by flow cytometry (FACS Canto, Becton Dickinson). Neutrophils were gated by forward scatter and side scatter characteristics and total CD88 expression measured.

3.5.1 Critically ill patients

Individual CD88 PE median fluorescence ranged from 825 – 3845 arbitrary units with a group median of 1852 arbitrary units.

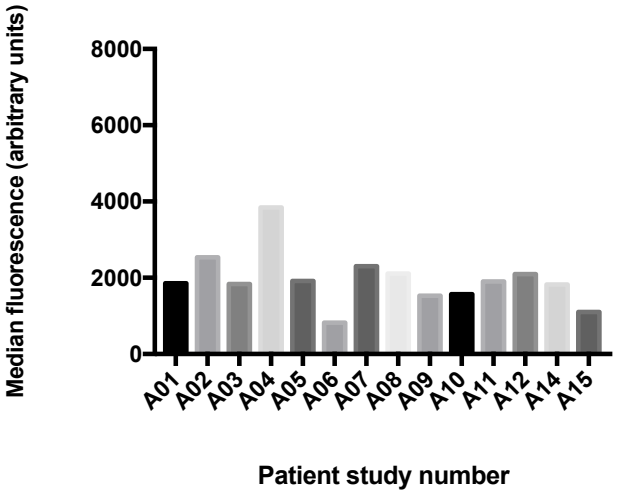

Figure 3d: Median neutrophil surface CD88 expression in critically ill patients.

3.5.2 Healthy volunteers

CD88 expression measurement was available for 7 of the nine healthy volunteers. 2 samples could not be analysed due to unavailability of the CD88 PE antibody. Individual CD88 PE fluorescence ranged from 2353 – 6258 with a group median fluorescence of 4305 arbitrary units.

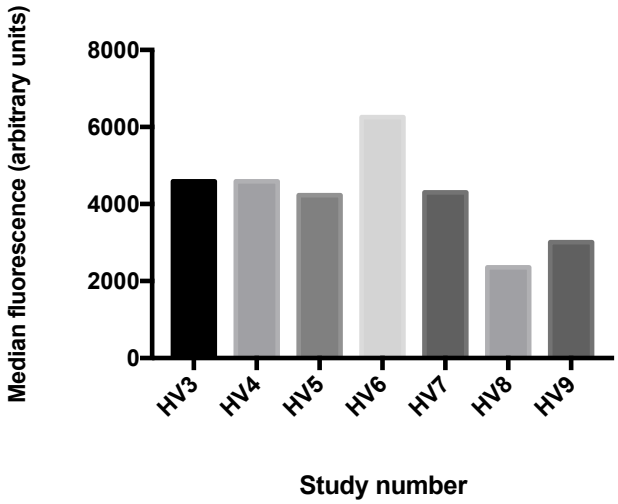

Figure 3e: Median neutrophil surface CD88 expression in healthy volunteers. Mean expression 4191 (SD+/- 1250) (arbitrary units).

3.5.3 Comparison between critically ill patients and healthy volunteers

There was a statistically significant difference in median CD88 PE expression between the two groups with lower expression among the critically ill patients.

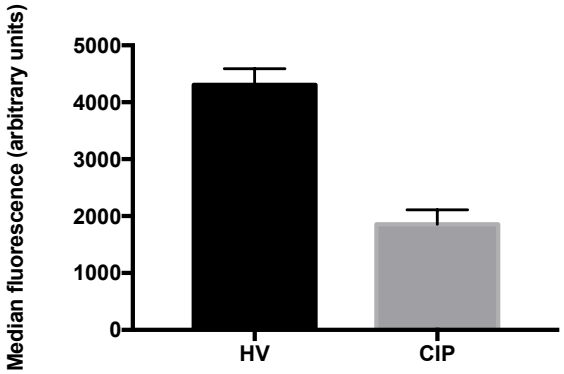

Figure 3f: Comparison between median neutrophil CD88 surface expression in critically ill patients and healthy volunteers. Critically ill median expression 1852 (IQR 1531-2108) vs Healthy volunteer median expression 4305 (IQR 3012-4591) (arbitrary units). P =0.0001. Mann Whitney U Test.

3.6 Correlation between neutrophil phagocytic capacity and neutrophil surface CD88 expression

The correlation between neutrophil phagocytic capacity and CD88 expression was then examined in an attempt to identify a CD88 level which would correspond to 50% phagocytic capacity and which could therefore potentially be used to identify patients eligible for the DFS and RCT.

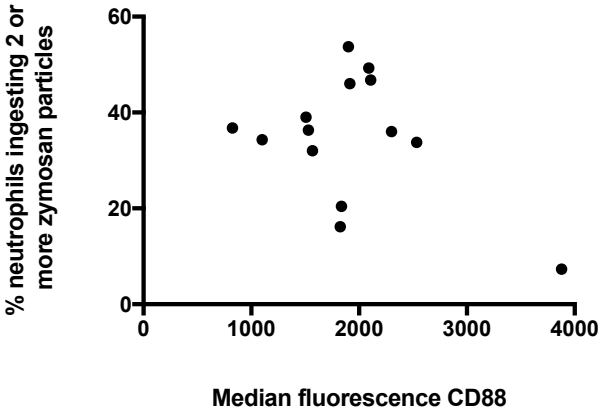

Figure 3g: Correlation between neutrophil surface CD88 expression and neutrophil phagocytic capacity amongst critically ill patients. (r=-0.07, p=0.82, Spearman's test).

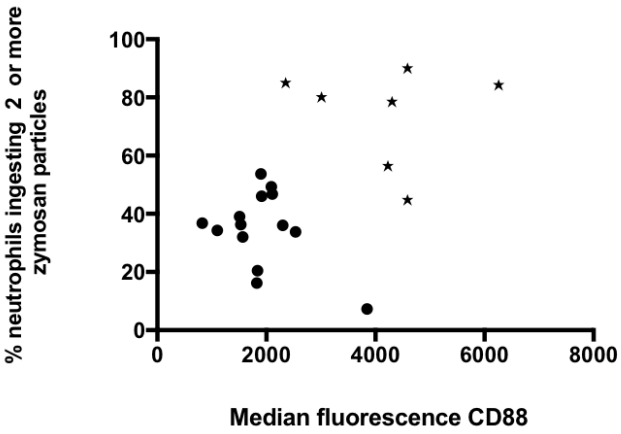

Figure 3h: Correlation between neutrophil surface CD88 expression and neutrophil phagocytic capacity. Pooled data for critically ill patients (black dot, n=14) and healthy volunteers (black star, n=7). (r=0.52, p=0.015, Spearman's test).

The was no correlation between neutrophil phagocytic capacity and CD88 surface expression when analysing either critically ill patients (r= -0.07) or healthy volunteers (r=0.14) alone. When data were pooled for critically ill patients and healthy volunteers, however, the correlation improved to moderate with a correlation co-efficient r=0.52 (p=0.015). The strength of the correlation was not in keeping with previous work suggesting a strong correlation between the two variables (Conway Morris et al., 2009, r=0.69, p=<0.001).

3.7 Monocyte HLA-DR Expression

As described in chapter 2 (section 2.15.5) monocyte HLA-DR expression was assessed using a commercial Quanitbrite^{TM} kit, and expressed as the median number of molecules of antibody per cell. Measurement of mHLA-DR expression provided additional confirmation of the presence of immunoparesis in our patient cohort.

3.7.1 Critically ill patients

Monocyte HLA-DR expression in the critically ill group of patients was relatively low with a median of 4,796 antibodies per cell (IQR 2760-6868).

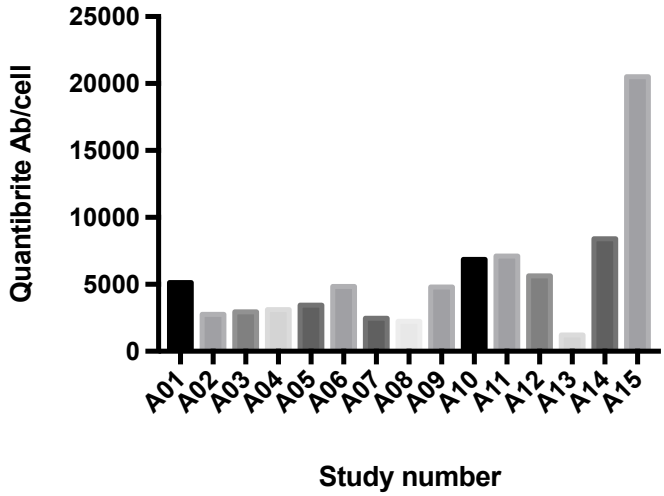

Figure 3i: Monocyte HLA-DR expression in critically ill ACS participants. Ab, antibodies. Median expression 4,796 Ab/cell (IQR 2760-6868), n=15.

3.7.2 Healthy volunteers

Amongst healthy volunteers, median monocyte HLA-DR expression was 25,699 (IQR 17,667 – 31,697). Analysis was lost in 1 patient.

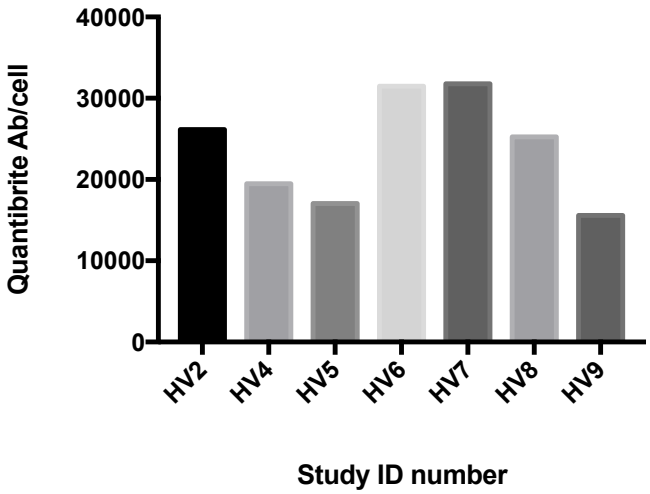

Figure 3j: Monocyte HLA-DR expression in healthy volunteers (HV).
Ab, antibodies. Median expression 25,699 (IQR 17,667-31,697), n=8.

Previous studies examining monocyte HLA-DR as a marker of immunosuppression have identified a cut-off of 5,000 antibodies per cell (Ab/cell) to identify those with immune paralysis and an increased risk of sepsis, following surgery. (Docke et al., 2005, Strohmeyer et al., 2003). A multicentre standardised study evaluating the use of BD Quantibrite^{TM} found a healthy reference standard value of ~25700 for the median number of HLA-DR antibodies per cell with 2.5th -97.5th percentiles of 14100-42500, with values in the range of 5000-15000 antibodies per cell in keeping with moderate to severe immunosuppression and greater than 15000 indicating immune competence. (Docke et al., 2005)

3.7.3 Comparison between critically ill patients and healthy volunteers

There was a statistically significant difference in the monocyte HLA-DR expression level between healthy volunteers and critically ill patients (p=<0.0001, Mann Whitney U Test), with the majority of the critically ill patients (9/15, 60%) falling into the category for immunoparalysis (<5000Ab/cell), 5 patients (33.3%) having evidence of

moderate to severe immunosuppression (5000 – 15000 Ab/cell) and only 1 patient (6.7%) having levels associated with immune competence (>15000 Ab/cell).

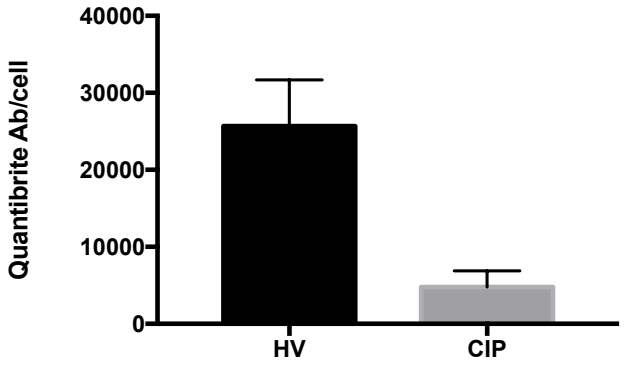

Figure 3k: Monocyte HLA-DR expression in healthy volunteers (HV) compared to critically ill patients. Critically ill median expression 4,796 Ab/cell (IQR 2,760-6,868) vs Healthy volunteer median expression 25,699 Ab/cell (IQR 17,667 – 31,697). p <0.0001. Mann Whitney U Test. Ab, antibodies

3.8 Correlation between monocyte HLA-DR expression and neutrophil phagocytic capacity

There was a similar correlation between monocyte HLA-DR expression and phagocytic capacity as there was between neutrophil CD88 expression and neutrophil phagocytic capacity with a Spearman's co-efficient of r=0.60.

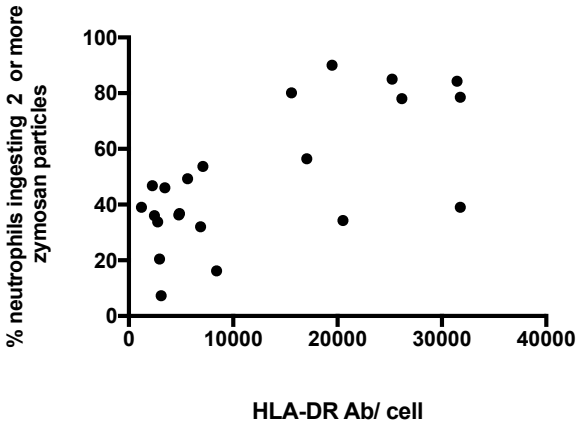

Fig 3l: Correlation between monocyte HLA-DR expression and neutrophil phagocytosis. Data from healthy volunteers (n=8) and critically ill patients (n=14) are pooled. r=0.55, p=0.008, Spearman's test.

3.9 Outcome

Patients were followed up at 30 days from collection of the blood sample to determine their outcome. 5 patients (5/15, 33.3%) had died during their admission to hospital: 4 whilst on ICU (4/15, 26.7%) and 1 following discharge to the ward. 1 patient remained an inpatient on ICU (1/15,6.7%). 8 further patients (8/15, 53.3%) remained in hospital but had been discharged from ICU to a ward. 1 patient (1/15,6.7%) had been discharged home.

Patient Study ID	Age (years)	Sex	Reason for admission	Outcome
A01	78	F	Oesophageal perforation	Discharged to ward
A02	85	M	Gallbladder empyema	Discharged to ward
A03	78	M	Femoral embolectomy / fasciotomy	Died on ICU
A04	61	M	Liver failure, GI bleed / cirrhosis	Died on ICU
A05	80	M	Community acquired pneumonia	Died on ward
A06	78	F	Pancreatitis	Discharged to ward
A07	62	M	Elective AAA post-op resp failure	Inpatient ICU
A08	31	F	Gallstone pancreatitis	Discharged to ward
A09	66	M	ALD, sepsis, renal failure	Died on ICU
A10	52	F	Cellulitis, renal failure	Discharged to ward
A11	71	F	Rhabdomyolysis	Discharged to ward
A12	38	M	Pancreatitis	Discharged home
A13	68	F	Bowel obstruction	Died on ICU
A14	65	F	Exacerbation COPD / CAP	Discharged to ward
A15	55	F	Asthma exacerbation	Discharged to ward

Table 13. Outcome data for ACS participants. AAA, abdominal aortic aneurysm; ALD, alcoholic liver disease; CAP, community-acquired pneumonia; COPD, chronic obstructive pulmonary disease; GI, gastrointestinal; ICU, intensive care unit; resp, respiratory

3.10 Discussion

The ACS aimed to confirm whether neutrophil CD88 expression could reliably be used as a surrogate marker for neutrophil phagocytic capacity in determining eligibility for the subsequent phases of the trial.

As expected neutrophil phagocytic capacity, CD88 expression and monocyte HLA-DR expression were all reduced in critically ill patients compared with healthy volunteers. The level of all 3 parameters was within the expected range for patients with critical illness-associated immune dysfunction, suggesting that we were successfully recruiting our target population to the study.

The assay confirmation study provided further valuable information that influenced the design of the subsequent phases of the trial in several areas.

3.10.1 Reliability of neutrophil CD88 expression as a marker of neutrophil phagocytic capacity

Within this patient cohort, in our laboratory, CD88 was not found to be a suitably reliable marker with which to identify patients with impaired neutrophil function who would be eligible for entry into the DFS and RCT.

The reason for the discrepancy between our own results and the previous results of Conway Morris et al is unclear however it may in some part have been due to a combination of the small sample size, the unmatched healthy volunteer population and differences in the clinical status of the recruited patient cohorts.

The ACS initially set out to recruit up to 30 patients however due to slow recruitment and time constraints an interim analysis was carried out after 15. The correlation coefficient of $r=0.52$ ($p=0.015$) was not felt to be adequate to ensure that CD88 expression could reliably determine eligibility for the study and therefore a decision was made to proceed to the DFS using the neutrophil phagocytic assay itself as the final determinant of eligibility, with only those participants with impaired neutrophil phagocytic capacity (i.e. < 50% neutrophils ingesting 2 or more zymosan particles) continuing.

The proportion of patients with adequate neutrophil phagocytic capacity was lower than expected when compared with Conway Morris' previous work where approximately 30% of critically ill patients retained satisfactory neutrophil phagocytic

capacity (Conway Morris et al., 2011). The finding that only 1 of the 14 (7.1%) patients, in whom phagocytic capacity was successfully assessed, had neutrophil phagocytic capacity ≥50% may reflect the fact that we had recruited a sicker population of patients. In Conway Morris' initial cohort patients were recruited following admission to intensive care if they were clinically suspected to have developed ventilator-associated pneumonia (VAP) (Conway Morris et al., 2009). This requires a patient to have been ventilated for at least 48 hours and in fact median duration of mechanical ventilation in these patients at the point of sampling was 8 days. There was a strong correlation between neutrophil CD88 expression and neutrophil phagocytic capacity with r=0.69 (Conway Morris et al., 2009). Compared to our study cohort, these patients were recruited and sampled at a different point in the natural history of their critical illness, having already developed a secondary infection, perhaps following a period of relative immunosuppression / paresis. The results may therefore not be directly comparable. Conway Morris went on to study a second cohort of patients, all of whom were recruited early in their admission to ICU with very similar inclusion and exclusion criteria to our own. In this cohort the correlation was once again demonstrated with r=0.80, suggesting that the timing of sampling and /or phase of critical illness may not be responsible for our failure to reproduce this strength of correlation between CD88 expression and phagocytosis (Conway Morris et al., 2011).

Our use of university staff and students as healthy volunteers meant that it was not possible to match our groups by age. As increasing age is known to have an effect on various aspects of immune function (Panda et al., 2009). it is possible that having a healthy control population unmatched for age may have an impact on the correlation between these two markers of immune function. In Conway Morris' first cohort of patients/healthy volunteers, the volunteers were recruited from a local general practice and were fully age and sex matched. In his second cohort, however, the healthy volunteer population was also recruited from university students and staff and therefore it is unlikely that these were age and sex matched any more closely than our own cohort, suggesting that this factor may not be of significant importance in terms of the correlation.

3.10.2 Suitability of inclusion and exclusion criteria

Through the screening process, during the ACS, it was recognised that the inclusion and exclusion criteria set to determine eligibility for the study were excluding some individuals, within the target population, who may stand to benefit from the intervention being studied. As discussed the ratio of patients screened to patients recruited was greater than 30:1.

In terms of the inclusion criteria the commonest reasons for patients not meeting the eligibility criteria were i) they did not require the support of one or more organ system and ii) they did not fulfil the criteria for SIRS. A significant proportion of patients on intensive care units have been admitted post-operatively for high level observation following elective surgery. These patient often do not require organ support and in addition may not meet the criteria for SIRS. In circumstances where their surgery has been uncomplicated such patients would be expected to make a steady recovery with early discharge from the intensive care unit. This cohort of patients accounted for a significant proportion of screened patients who were found to be not eligible for participation in the study. Such patients were not in the intended target population and therefore appropriately did not meet the inclusion criteria.

A much smaller proportion of patients did not meet the eligibility criteria because they did not fulfil the defined criteria for SIRS, despite being critically ill and requiring organ support. It has been recognised for quite some time that the SIRS criteria lack both sensitivity and specificity in identifying critically ill patients with sepsis. The SIRS criteria focus on the pro-inflammatory response and pre-date the accepted understanding that sepsis is a highly complex immune state involving dysregulated pro and anti-inflammatory pathways (Singer et al., 2016). A recently published study conducted in Australia and New Zealand, including more than a million patients, identified that 1 in 8 patients with severe sepsis and organ dysfunction admitted to ICUs do not meet the criteria for SIRS despite having a comparable mortality rate to those individuals who do (Kaukonen et al., 2015). As at the time of designing the study the SIRS criteria were still widely accepted as a useful indicator of eligibility for trials in critical illness we did not opt to alter this criterion. Since that time the sepsis criteria have been further revised, with the publication of Sepsis 3, and no longer include the SIRS criteria reflecting the improved understanding of the pathophysiology of sepsis (Singer et al., 2016).

The exclusion criteria had been set with extreme attention paid to safety and the potential risk of harm to patients. Following the recognition that a significant number of critically ill patients who may have stood to benefit from the intervention were being excluded on the basis of the stringent exclusion criteria a review of the existing criteria was made in advance of proceeding to the DFS and RCT. After careful consideration, revisiting the relevant literature and seeking advice from independent experts, it was agreed between the Trial Steering Committee and the Ethics Committee that some of the criteria should be revised in order to ensure recruitment of the target population. It was felt that the proposed alterations to the eligibility criteria would not pose any increased risk to patients entering the study but would enable patients to be appropriately recruited.

Revision of the following exclusion criteria took place prior to the commencement of the DFS.

i) Haemoglobin <8.5g/dL at the time of screening – this criterion had been set to ensure that patients would not experience clinically significant anaemia as a consequence of alternate day or daily blood sampling during the DFS and RCT. Many critically ill patients have anaemia as a consequence of their critical illness, however current critical care guidelines recommend that blood transfusion only be administered if the haemoglobin level falls below 70 g/L, aiming for a target haemoglobin following transfusion of 70-90 g/L. (Retter et al., 2013) After consultation with a local independent Consultant Haematologist we were reassured that the volumes of blood being collected during both the DFS and RCT phases of the study would not significantly impact on the haemoglobin level of participants and that it was therefore safe and ethical to lower the cut-off for entry to 7.5g/dL.

ii) Total white cell count > $20x10^9$/ litre at the time of screening – this criterion had been set to ensure that patients would not have a clinically significant rise in their white cell count following administration of GM-CSF to a level whereby they may suffer harmful consequences of leucocytosis. A significant proportion of patients with sepsis and non-septic critical illness experience a rise in their white cell count in excess of $30x10^9$/L. Again following advice from an independent Consultant Haematologist, it was agreed that this criterion could be safely increased to $30x10^9$/litre without increasing the risk of significant adverse events secondary to

leucocytosis including leukostasis, which is usually seen with WBC counts of greater than $100x10^9$/L. (Ganzel et al., 2012)

The in-hospital mortality rate of 33.3% and ICU mortality rate of 26.7% in the cohort of patients in this phase of the study are in keeping with expected mortality for ICU patients suffering sepsis and critical illness (Vincent J-L et al., 2006) and represent the expected mortality for our target population in the subsequent phases of the trial.

3.10.3 Effects of critical illness on the neutrophil separation technique

During the isolation of neutrophils, it was noted that the percoll gradient separation of leucocytes in patients with critical illness was less well defined than in healthy volunteers with a much broader neutrophil band being present which on occasions was seen to merge with the band of isolated mononuclear cells. This stage of neutrophil extraction relies on the separation of cells on the basis of their relative density, with mononuclear cells appearing at the 55%/70% layer and neutrophils appearing at the 70%/81% layer (Chapter 2, section 2.16.1). We postulated that the presence of increased fractions of immature neutrophils, being released from the bone marrow in response to systemic inflammation, may result in less distinction between the cell populations in terms of their densities. Immature neutrophils are larger and less dense then fully matured neutrophils and may have been responsible for the band just below the mononuclear cell population, which was often seen to merge with the usually distinct mature neutrophil population. Harvesting of the mononuclear cell layer revealed contamination with what morphologically appeared to be immature neutrophils.

Similar findings have been reported in studies of both animal and human neutrophils in a variety of pro-inflammatory conditions. What have been termed as low-density neutrophils or granulocytes (LDN/ LDGs) have been found in a range of disease states including sepsis (Morasaki et al., 1992), systemic lupus erythematosus (SLE) (Carmona-Rivera et al., 2013), human immunodeficiency virus infection (Cloke et al., 2012) and cancer (Sagiv et al.,2014).

Hacbarth et al. were the first to report the finding of low-density neutrophils contaminating the mononuclear layer of Ficoll-Hypaque gradient separation samples in patients with SLE and rheumatoid arthritis. They described these cells as low buoyant density neutrophils and demonstrated that normal density healthy

neutrophils could be altered to become low-density neutrophils by the addition of serum from an affected patient or through complement activation by addition of inulin to autologous serum. In these circumstances the proportion of low-density neutrophils found in the mononuclear cell layer increased significantly (Hacbarth et al., 1986). Hacbarth et al hypothesised that the reason for the low density in these cells was a result of degranulation with an associated increase in cell volume. They reported that the neutrophils found in the mononuclear cell layer were mature neutrophils, however they did not comment on how this was confirmed or whether there was also evidence of immature neutrophils within the neutrophil population. They excluded the possibility of cell aggregation being the reason for an increase in buoyant neutrophils via light microscopy. Subsequent work by Denny et al aimed to characterise these LDNs in more detail. They showed that these cells had a similar activation status to both normal density granulocytes in the same patients and neutrophils from healthy controls. The LDNs had a reduced capacity for phagocytosis but an enhanced ability to produce neutrophil extra-cellular traps (NETs). In addition, the researchers carried out ultra-structural analysis of the LDN population, with transmission electron microscopy, which demonstrated nuclei compatible with a range of immature neutrophil subsets and evidence of intact intracellular granules (Denny et al., 2010). Contrary to the findings of Hacbarth et al, these findings suggest that LDNs represent immature cells within the blood rather than cells that have degranulated.

Pember et al carried out in-vitro and in-vivo studies of LDNs in experimental sepsis. Neutrophils harvested from mice and humans showed a wide band of density distribution following activation compared to controls. Stimulation of high-density neutrophils with endotoxin-activated serum induced a shift in cell density to lower levels. A similar effect was seen in human neutrophils exposed to fMLF where the reduction in density was associated with an increase in mean cell volume. Cells prompted to de-granulate also showed a shift to a lower density but to a lesser extent than those exposed to LPS. (Pember et al., 1983)

A Japanese study in patients with sepsis subdivided isolated neutrophils into high-density, intermediate-density and low-density cells and compared the relative proportions of each cell type in the septic cohort against healthy controls. The predominant cell type in the healthy controls was high-density neutrophils with a

mean of 76 +/- 9%. Patients with evidence of moderate infection had an increased proportion of intermediate-density neutrophils whilst in those with severe infection low-density neutrophils made up 40% of the total neutrophil population (Morasaki et al., 1992). Further functional analysis of the cell density subtypes showed a reduction in chemotactic response in low-density neutrophils compared to high-density neutrophils in both septic patients and healthy volunteers. Electron microscopy of the low-density neutrophils from the septic cohort showed evidence of degranulation and the presence of vacuoles, leading the researchers to conclude that the increased proportion of LDNs seen in septic patients was secondary to degranulation of high density neutrophils (Morasaki et al., 1992).

Recent reviews suggest that no single factor is responsible for the appearance of LDNs and that the presence of immature neutrophils, activated degranulated neutrophils and granulocytic myeloid-derived suppressor cells (G-MDSCs, seen in cancer and HIV), may all contribute to the LDN population in varying proportions depending on the disease state (Scapini et al., 2010).

As we wished to study neutrophil function as a whole in critically ill patients we collected the entire neutrophil population from the whole bandwidth in each case. In practice, the absence of a clear band of separation between the neutrophil and mononuclear cell populations undoubtedly results in the unavoidable loss of a portion of the neutrophil population during harvesting, in order to ensure purity of the neutrophil population. Review of the literature, however, did not reveal any recommendation for an alteration in the composition of the percoll gradient in such circumstances.

3.10.4 Effects of heparin on preparation of serum

During the processing of patient whole blood samples, following centrifugation, the platelet-rich plasma was transferred to a glass serum bottle and $CaCl_2$ added. The sample was then incubated in a water bath at 37°C until a platelet clump had formed. Autologous serum was then collected for use in subsequent phagocytosis assays.

It was noted that in several of the samples from critically ill patients a platelet clump failed to form despite extended incubation with $CaCl_2$. Review of these patients found that they were receiving therapeutic doses of systemic heparin therapy for a variety

of indications including continuous veno-venous haemofiltration and anticoagulation of a Novalung extracorporeal CO_2 removal circuit.

It was acknowledged that therapeutic anticoagulation with heparin was likely to affect a significant proportion of the patient samples collected during the DFS and RCT in view of the nature of the participants' underlying illnesses. Reversal of the anticoagulant effects of heparin can be achieved through the administration of protamine sulphate.

Both heparin and protamine sulphate have been shown to have an effect on neutrophil phagocytosis, with heparins having an inhibitory effect and protamine a stimulatory effect. (Salih et al., 1997, Peterson et al., 1984.)

In view of the fact that the purpose of the trial was to assess the potential for GM-CSF as a therapy for critically ill patients it was agreed that alteration of the participants' serum profile with, for example, protamine to reverse the effects of heparin may produce a misleading result in terms of real-life clinical application. For the same reason it was felt inappropriate to exclude patients receiving anticoagulation. It was therefore decided that 2 mL of the original 20mL whole blood sample would be collected into a serum gel tube to allow preparation of autologous serum for use in subsequent neutrophil functional assays.

3.11 Limitations of the Assay Confirmation Study

The main limitations to the ACS were in relation to the sample size and the lack of matching in the healthy volunteer comparison cohort.

3.11.1 Sample Size

As discussed the ACS initially set out to recruit up to 30 patients. Rate of recruitment was significantly below target recruitment, in part related to the stringent entry criteria. Over a period of 4 1/2 months, 15 patients were recruited from 3 ICUs. Interim analysis of the correlation between neutrophil phagocytosis and neutrophil surface CD88 expression did not suggest a strong enough correlation to advise use of neutrophil surface CD88 expression as a surrogate marker for neutrophil phagocytic capacity to determine final eligibility for entry into the study. As there was a suggestion of a moderate correlation it was felt that this relationship warranted further investigation in the DFS and RCT phases of the study. The aim of the ACS

was to determine the validity of CD88 expression as a surrogate marker for neutrophil phagocytic capacity however the strength of the correlation may have been under estimated by insufficient numbers of patients being included in this phase of the study.

3.11.2 Unmatched healthy volunteers

Because our cohort of healthy volunteers came from a university population of staff and students we were unable to match basic demographic data in particular in terms of age. Increasing age is known to be associated with alterations in innate immune function and therefore differences in two groups unmatched for age may not reflect simply the presence of critical illness in the patients. (Berrut et al. 2015). There is no available literature looking directly at the effect of age on neutrophil CD88 expression in adults. Any correlation between these two parameters may be age-dependent and may therefore have affected our results.

3.12 Conclusions of Assay Confirmation Study

In conclusion the ACS did not confirm a strong enough correlation between neutrophil CD88 expression and neutrophil phagocytic capacity for CD88 to be used as a surrogate marker to confirm eligibility for the study. In the process of this assessment several issues were highlighted in relation to the inclusion and exclusion criteria for the study requiring modification. On completion of the ACS minor modifications were made to the inclusion and exclusion criteria for the DFS and RCT following which recruitment to the DFS commenced.

Chapter 4: Dose Finding Study.

This chapter will outline the results of the dose finding study. It will begin by stating the aim of the study, give details regarding the conduct of the study, describe the screening, recruitment and consent process, and will then go on to report the results.

4.1 Aim of study

The aim of the dose finding study was to establish the optimum dose and duration of GM-CSF which would demonstrate evidence of a biological response in terms of improvement in phagocytosis alongside a favourable safety profile. This dose would then be carried forward for use in the randomised controlled trial.

4.2 Study Design

The dose finding study was designed to test 2 doses of GM-CSF (3µg/kg/day and 6µg/kg/day, for 2 lengths of administration (4 and 7 days), in 4 sub-groups of patients. 6 patients were planned to be recruited to each sub-group of dose/duration. Sample size for the dose finding study was derived pragmatically on the basis that similar numbers of participants in similar situations have previously provided meaningful results. A sample size of 6 per group (24 in total) was calculated to give a power of 80% power to detect an effect size of 1.8 with a probability of 0.05, between any 2 treatment groups, using a 2 sample t-test.

Low dose GM-CSF (3µg/kg/day) would be trialled for 4 days in the first instance (patients 1-6) followed by seven days (patients 7-12), after which the dose would be doubled (6µg/kg/day) and trialled for the same two durations of treatment; 4 days (patients 13-18), 7 days (patients 19-24). (Table 14).

Following completion of the Assay Confirmation study it was recognised that some of the inclusion and exclusion parameters were excluding patients within the target study population. As discussed in chapter 3 minor modifications to the inclusion and exclusion criteria were therefore made and approved by the relevant regulatory bodies prior to commencing enrolment to the dose finding study;

I. The cut off for haemoglobin was lowered to 7.5g/dL

II. The cut off for white cell count was raised to $30x10^9$/L

In addition, it was observed that the correlation between neutrophil CD88 expression and neutrophil phagocytic capacity was not as strong in the ACS cohort of patients as in the previous work carried out by Conway Morris et al. It was therefore decided to use neutrophil phagocytic capacity itself as the final eligibility criterion for entry into the dose finding study and randomised controlled trial and to continue to explore the potential use of neutrophil CD88 expression as a surrogate marker for phagocytic capacity during these final stages of the trial. The dose finding study protocol is found in Appendix 1.

Patient Enrolment Number	Dose of Leukine (Sargramostim, rhu GM-CSF)
1-6	3 µg/kg/day for 4 days
7-12	3 µg/kg/day for 7 days
13-18	6 µg/kg/day for 4 days
19-24	6 µg/kg/day for 7 days

Table 14. GM-CSF dosing schedule for participants in Dose Finding Study

4.3 Study outcomes

4.3.1 Primary outcome

The primary outcome for the DFS was neutrophil phagocytic capacity on day 2 post-administration of GM-CSF

4.3.2 Secondary Outcomes

The study included several secondary outcomes

I. Neutrophil CD88 expression after administration of GM-CSF
II. Monocyte HLA-DR expression after administration of GM-CSF
III. Serum GM-CSF level after administration of GM-CSF
IV. Safety analysis including blood profile and PaO_2:FiO_2

4.4 Study conduct

To ensure safety during the administration of GM-CSF, following discussion with the DMSC, it was agreed that the first three patients would be recruited one at a time during the dose finding study with a brief report submitted to the DMSC following completion of each patient's drug dosing and blood sampling follow-up. The report included data regarding daily phagocytic capacity, blood parameters, safety analysis and a description of the clinical course followed. The data were reviewed by the DMSC prior to any additional patients being recruited. If the DMSC were satisfied that there were no issues of concern in relation to safety they would confirm that the trial could continue. This decision regarding single sequential recruitment was made particularly in light of previous concerns over the possible link between colony stimulating factors including GM-CSF and acute lung injury (ALI) and the adult respiratory distress syndrome (ARDS). (Goodman et al., 1999) In view of the fact that all patients recruited to the trial would by definition have critical illness the risk of development of ALI/ARDS, independent of GM-CSF therapy, was high. (Rubenfeld et al., 2007). Data were collected on PaO2:FiO2 pre- and post-drug administration to specifically look for evidence of this occurrence.

4.5 CONSORT Diagram

The CONSORT diagram below outlines the screening and recruitment of patients to the dose finding study.

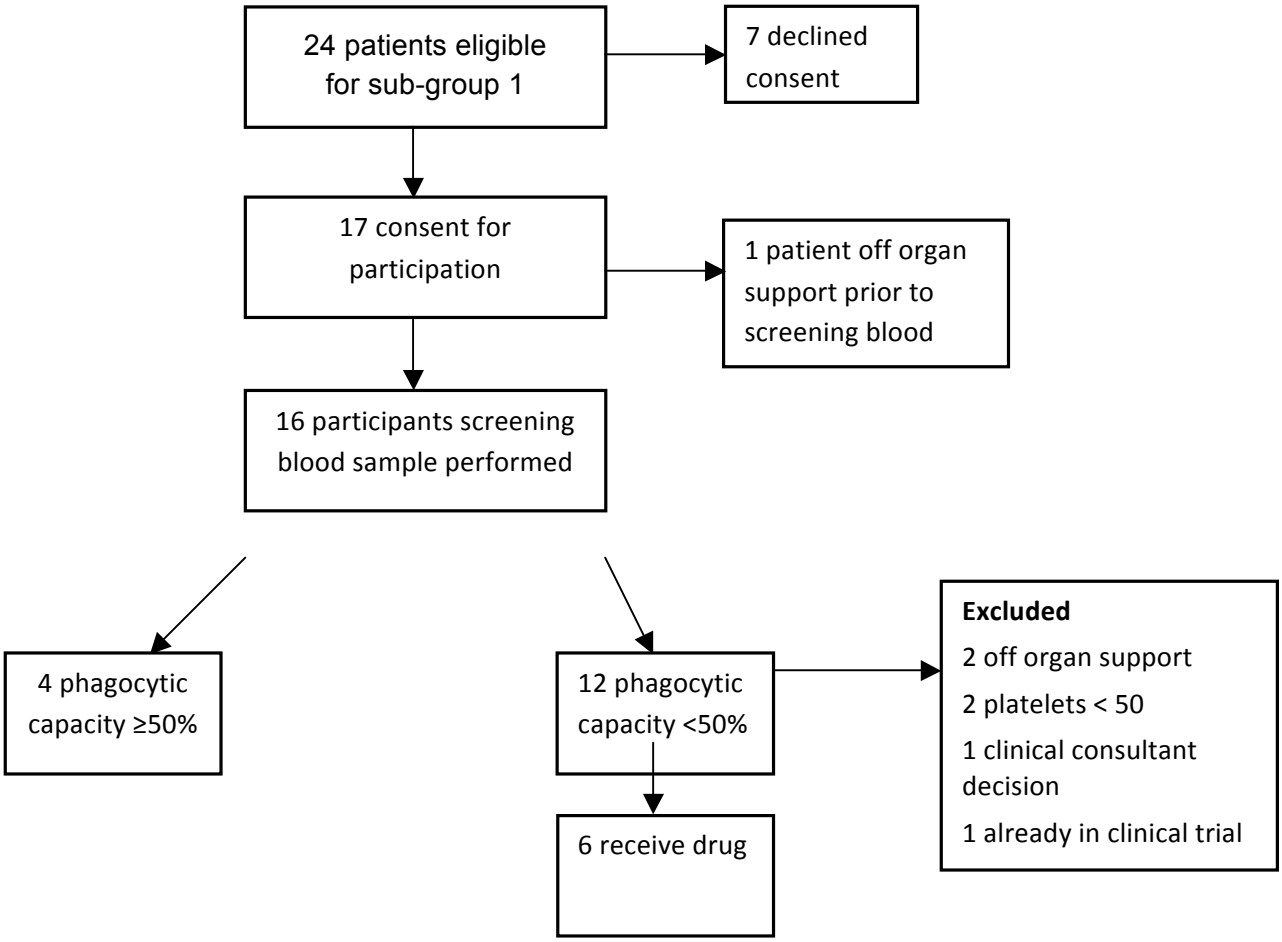

Figure 4a: Dose finding study CONSORT diagram. (only sub-group 1 was completed)

4.6 Recruitment

The DFS commenced on 5th January 2013 with the first patient being recruited to the study on 8th January 2013. All admissions to each intensive care unit were screened on a daily basis during the working week for eligibility for inclusion in the trial. Almost 1000 screening episodes took place. Screening did not take place on a weekend when there was no researcher available. As discussed above (section 4.2) we planned to recruit up to 24 patients to the dose finding study with 6 patients recruited to each dose-duration sub-group. 24 of the patients screened were found to be eligible for participation in the first phase (3μg/kg/day) of the Dose Finding Study. There were 7 instances of declined consent (2 by patients and 5 by personal legal

representatives). 17 patients or personal or professional legal representatives gave consent for participation in the study. The final patient was recruited on 30th July 2013.

4.7 Final eligibility

The final step of the eligibility assessment occurred on day 0 when blood was collected for assessment of neutrophil phagocytic capacity. Participants only proceeded at this stage if they were confirmed to have evidence of neutrophil dysfunction with neutrophil phagocytic capacity < 50%. Of the 17 patients who gave consent to participate in the study 11 were excluded from the study at this stage prior to receiving GM-CSF (4 patients had phagocytic capacity ≥ 50%, 3 patients had discontinued organ support, 2 patients had platelet counts less than $50x10^9$/L, 1 decision was made by a clinical consultant that the patient should no longer continue in the trial and 1 patient was found to be already enrolled in an interventional clinical trial which therefore precluded their participation). 6 patients (R01, F01, R02, Q03, R04, Q06) went on to receive GM-CSF as part of group 1 in the dose finding study.

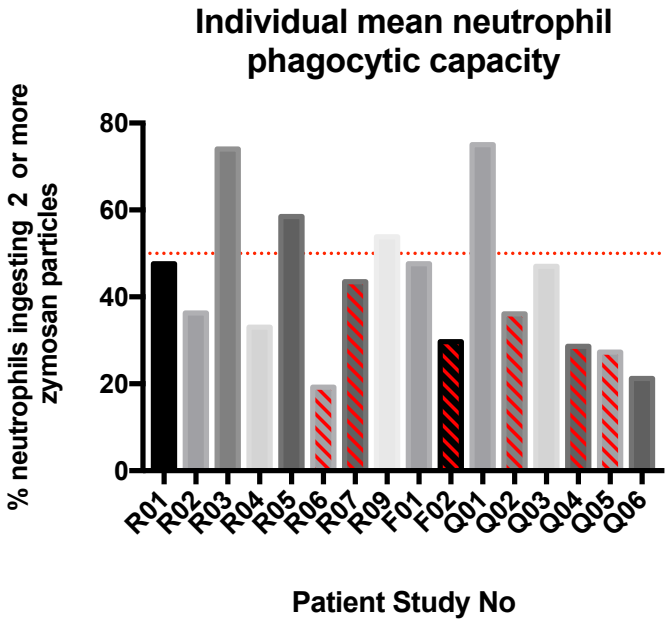

Figure 4b: Baseline individual mean phagocytic capacity in critically ill patients recruited to Dose Finding Study. Patients with neutrophil phagocytic capacity ≥ 50% (dotted red line) were excluded from the study (n=4). Patients excluded for other reasons (red hatching); 2 discontinued organ support, 2 platelet count less than 50, 1 decision made by clinical consultant ,1 already enrolled in an interventional clinical trial.

4.8 Baseline demographics of patients enrolled into the Dose Finding Study

The patient population recruited was broadly representative of a typical ICU cohort of patients. The cohort of patients proceeding past the final eligibility step was younger than those who were excluded at that stage.

Baseline demographic	**Treatment group phagocytosis <50% (n=6)**	**Excluded group phagocytosis >50% (n=4)**
Sex (M:F)	3:3	1:3
Age: Median (years, range)	56.5 (25-66)	70.8 (58 – 79)
Admission diagnosis	Community acquired pneumonia (CAP) Anastomotic leak Clostridium difficile colitis Bacterial endocarditis Oesophageal perforation Small bowel obstruction	Respiratory failure post burns CAP / acute kidney injury Pedestrian polytrauma Exacerbation COPD
APACHE II Score Median (range)	19.5 (13-25)	16 (12 – 22)
Weight Median (kg, range)	95 (56 - 144)	N/a
SOFA Score Median (range)	9.5 (2-14)	N/a
PaO_2:FiO_2 kPa (best prev 24hours)	37.7 +/- 19.2 (16.3 – 69.7)	N/a
WBC $x10^9$/L Mean +/- s.d. (range)	13.06 +/- 4.53 (8.91 – 21.1)	N/a
Neutrophils $x10^9$/L Mean +/- s.d. (range)	10.8 +/- 9.85 (6 – 18.7)	N/a

Table 15. Demographic data for participants in Dose Finding Study

CAP, community-acquired pneumonia; COPD, chronic obstructive pulmonary disease; APACHE, acute physiology and chronic health evaluation; SOFA, sequential organ failure assessment; WBC, white blood cell; s.d., standard deviation; PaO_2, partial pressure of oxygen; FiO_2, inspired concentration of oxygen

4.9 Study drug administration

All patients entering this phase of the study received a daily injection of subcutaneous GM-CSF at a dose of 3 microgram / kg /day. Daily administration of the drug was continued until any one of the study drug termination criteria was met:

- maximum treatment period
- study drug-related serious adverse reaction
- discharge from a critical care environment
- death
- discontinuation of active medical treatment
- the patient, PerLR or ProfLR requests withdrawal from the study
- decision by the attending clinician that the study drug should be discontinued on safety grounds

There were 2 situations whereby the protocol decreed that consideration should be given to either discontinuing the administration of GM-CSF or reducing the dose by half:

- WCC > 50,000 cells/mm^3
- Platelet count > 500,000 cells/mm^3

(Trial protocol, appendix 1).

GM-CSF was prescribed according to actual body weight up to a maximum weight of 120kg, corresponding dose of 360 µgm. Doses were prescribed within weight ranges to the nearest 5 kg. Median weight was 95kg with a range of 56 – 144kg and corresponding doses ranged from 165mcgm daily to 360mcgm daily. Above 1ml volume (255µg) doses were rounded to the nearest 0.1 of a ml. Five of the six patients received all four prescribed doses from day 0 to day 3. One patient did not receive their final dose on day 3 due to a fall in platelet count on this day. As GM-CSF is known to affect platelet count this triggered the study drug termination criteria above and a decision was made by the attending clinician to withhold the study drug on the grounds of ensuring patient safety.

4.10 Blood sampling

As described in chapter 2 blood samples were collected from patients each morning from D0 to D9. Blood samples for phagocytosis and other assessments of neutrophil function were not collected at weekends when no researcher was available. Blood samples for safety analysis including full blood count, urea and electrolytes and liver functions were collected everyday, including weekends, as part of usual clinical care.

4.11 Results

4.11.1 Neutrophil phagocytic capacity

4.11.1.1 Baseline phagocytic capacity

Neutrophil phagocytosis was measured at baseline (day 0, prior to administration of GM-CSF) and then on each day thereafter up to day 9. Mean baseline phagocytosis on day 0 was 38.7% (SD+/-10.6, range 21.2% - 47.6%).

4.11.1.2 Neutrophil phagocytic capacity on day 2.

The phagocytosis assay failed for 1 patient (R01) on day 2 due to lysis of cells at the final wash with PBS. This was thought to be due to contamination of the PBS solution. Therefore, no phagocytic capacity result was available for that patient on that day. All 5 other patients were found to have an improvement in their neutrophil phagocytosis on day 2 post administration of GM-CSF (the day for assessment of the primary outcome). In addition, all 5 patients with available data had a neutrophil phagocytic capacity ≥50% on day 2 following administration of GM-CSF. Mean neutrophil phagocytic capacity on day 2 post administration of GM-CSF was 59.6% (SD +/- 7.9) compared with neutrophil phagocytic capacity 38.7% (SD +/- 10.6) at baseline (p=0.06).

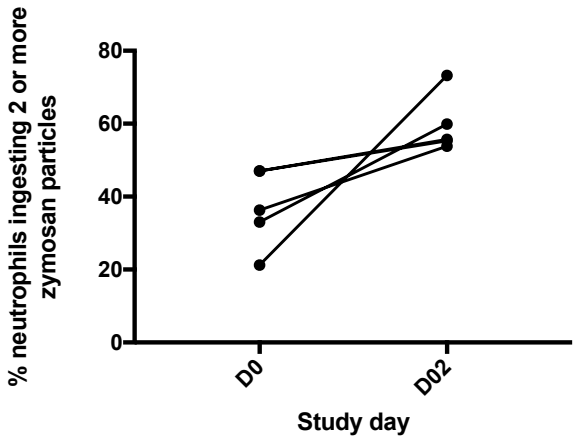

Figure 4c. Change in individual mean neutrophil phagocytosis between day 0 and day 2. GM-CSF improves neutrophil phagocytosis by day 2. Percentage neutrophils ingesting 2 or more zymosan particles (n=5). Note two patients results overlapping.

Although not part of the formal analysis plan, when imputation by mean for the dataset (59.6) or minimum for the dataset (53.8) substitution was applied for missing variable R01 D2 there was a statistically significant difference in mean phagocytosis at day 2. (Mean substitution p=0.03, minimum substitution p=0.03).

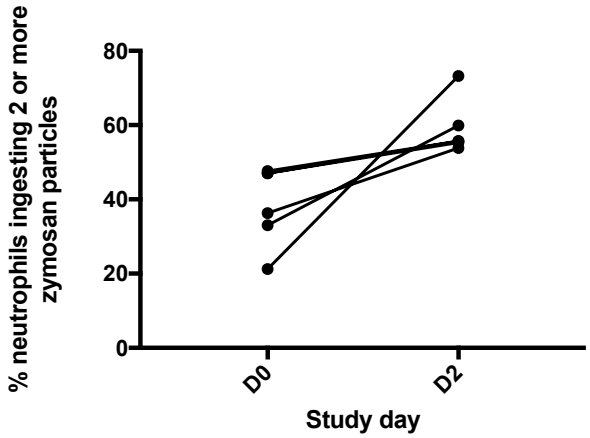

Figure 4d. Change in individual mean neutrophil phagocytosis between day 0 and day 2 GM-CSF improves neutrophil phagocytosis by day 2. Mean substitution for missing variable R01 D2 (p=0.03 by Wilcoxon matched pairs rank test) Mean difference 20.9%, median difference 15.5% between day 0 and day 2 (n=6). Note 3 patients results overlapping.

4.11.1.3 Neutrophil phagocytic capacity over study period.

Improvement in mean neutrophil phagocytic capacity was seen to persist at day 7 (last day where all 6 patients were sampled) however did not reach statistical significance. Overall mean neutrophil phagocytic capacity was 65% (SD+/-9.2) on day 7 compared with 38.7% (SD+/-10.6) on day 0 (p=0.06). In one patient (Q03) after an initial improvement in neutrophil phagocytic capacity on day 2, the neutrophil phagocytic capacity was seen to fall until day 7 rising again on days 8 and 9.

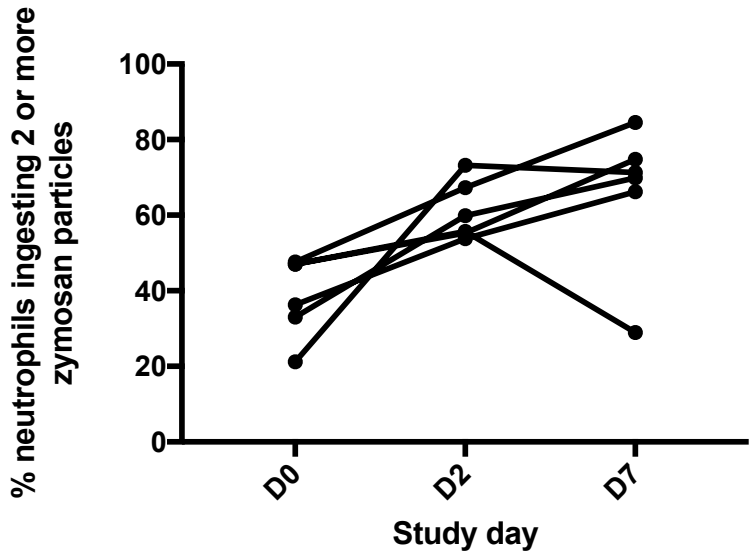

Figure 4e. Individual mean neutrophil phagocytosis over study period. The trend towards improvement in neutrophil phagocytic capacity persists at Day 7 but did not reach statistical significance (p=0.0625 by Wilcoxon matched pairs rank test comparing D0 and D7) Mean diff 26.9%, median diff 28.38% D0 – D7.

4.11.1.4 Summary descriptive statistics for neutrophil phagocytic capacity days 0, 2 and 7.

Mean neutrophil phagocytic capacity rose by 20.9% between day 0 and day 2 and 27.2% between day 0 and day 7.

Statistical Value	D0	D2	D7
No of values (n)	6	5	6
Minimum	21.2	53.8	29.0
25% percentile	30.1	54.6	56.9
Median	42.2	55.7	70.6
75% percentile	47.1	66.6	84.5
Maximum	47.6	73.2	77.2
Mean	38.7	59.6	65.9
Sd	10.6	7.9	19.2
S.E.M	4.3	3.6	7.8
Lower 95% CI	27.6	49.7	45.8
Upper 95% CI	49.8	69.4	86.0

Table 16. Summary descriptive statistics for neutrophil phagocytic capacity days 0, 2 and 7.

4.12 Monocyte HLA-DR Expression

4.12.1 Baseline monocyte HLA-DR expression

As described in chapter 2 monocyte HLA-DR was measured at baseline and daily on weekdays in all patients by flow cytometry using the Quantibrite™ kit. Baseline monocyte HLA-DR expression was low with 5 of the 6 participants having monocyte HLA-DR level below 15,000 Ab/cell in keeping with moderate to severe immune dysfunction. 3 of these 5 patients had mHLA-DR levels below 5000 Ab/cell in keeping with immunoparalysis. Only 1 patient had a HLA-DR level greater than 20,000 Ab/cell suggestive of immune competence. Median mHLA-DR at baseline was 4297 Ab/cell (range 2852-10100Ab/cell).

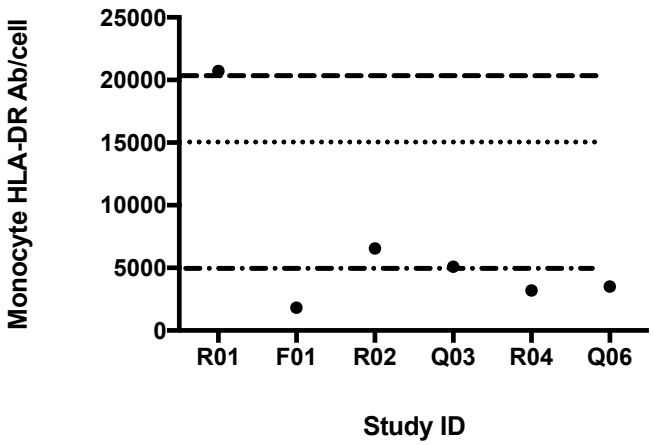

Figure 4f. Individual monocyte HLA-DR expression at baseline. Measurement performed by Quantibrite™ analysis. Median mHLA-DR 4297 Ab/cell (2852 – 1911 Ab/cell).

4.12.2 Monocyte HLA-DR expression over study period

Following administration of GM-CSF there was a general trend towards an increase in monocyte HLA-DR expression with a rise being seen in all but one patient. Median mHLA-DR D0 - 5091 (3348-13642) vs D2 - 25881 (14722-64300); vs D3 – 27599 (17002-31710); vs D5 – 13255 (3681-24561); vs D7 – 4603 (2530-6786); vs D9 – 4387 (3036-8792). Following completion of the study drug on D3 mHLA-DR levels were seen to fall. In all cases, by study day 9, mHLA-DR had fallen to levels previously seen in patients with at least moderate immunosuppression i.e. less than 15,000 Ab/cell.

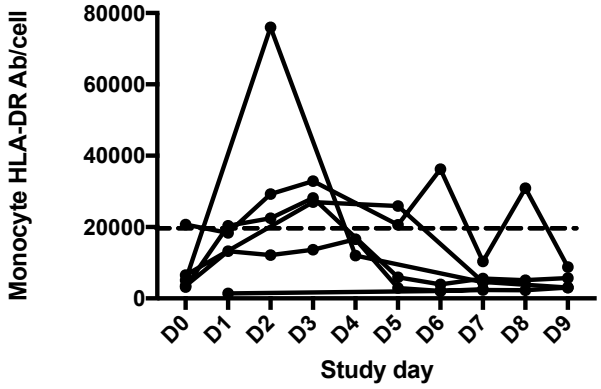

Figure 4g. Individual monocyte HLA-DR expression over study period

4.12.3 Correlation between monocyte HLA-DR expression and neutrophil phagocytic capacity

A weak correlation was seen between baseline mHLA-DR (D0) and neutrophil phagocytic capacity (Spearman's r = 0.41.)

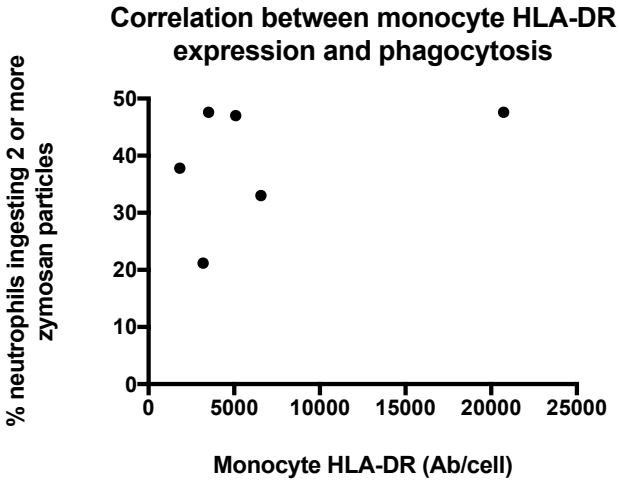

Figure 4h. Correlation between baseline monocyte HLA-DR expression and neutrophil phagocytic capacity. (Spearman's r = 0.41, p = 0.15, n=6)

There was no correlation seen between mHLA-DR and neutrophil phagocytic capacity following the administration of GM-CSF.

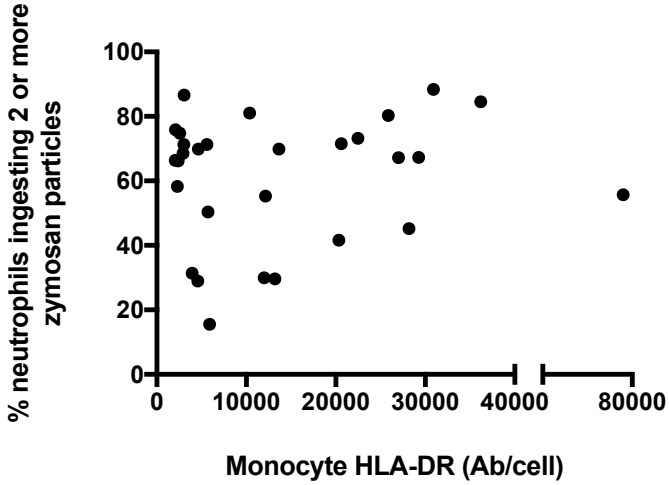

Figure 4i. Correlation between m HLA-DR expression and neutrophil phagocytic capacity following GM-CSF administration. (Spearman's r = 0.07, p = 0.72, n=29)

4.13 Neutrophil CD88 Expression

4.13.1 Baseline neutrophil CD88 expression

Mean baseline neutrophil CD88 expression was 830 arbitrary units (s.d. +/- 304.5), median 778.

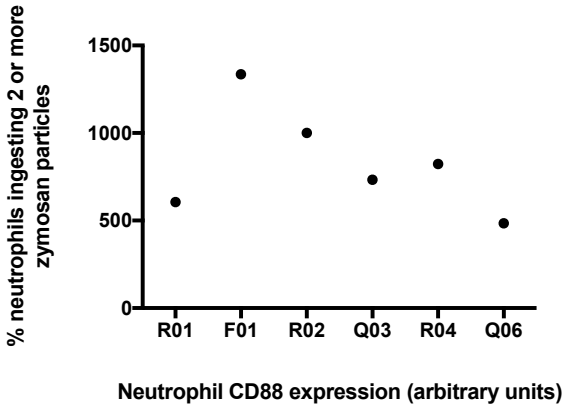

Figure 4j. Individual neutrophil CD88 expression at baseline. Mean neutrophil CD88 expression was 830 (s.d. = +/- 304.5, median 778 (576-1085).

4.13.2 Neutrophil CD88 expression over study period

Following administration of GM-CSF there was a general trend towards an increase in neutrophil CD88 expression seen in all patients.

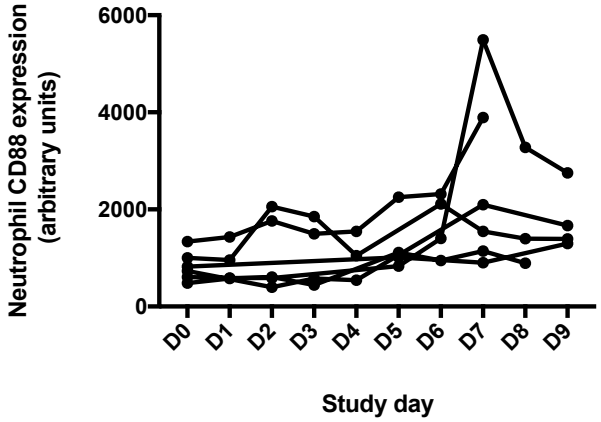

Figure 4k. Individual neutrophil CD88 expression over study period. Median neutrophil CD88 expression (arbitrary units) was as follows - D0 778 (575 – 1085) vs; D2 608 (493 – 2057), D3 1037 (476 – 1764), D5 1062 (879 – 1967), D7 1821 (1080 – 4292), D9 1530 (1322 – 2481).

4.13.3 Correlation between neutrophil CD88 expression and neutrophil phagocytic capacity

A strong correlation was observed between baseline neutrophil CD88 expression and neutrophil phagocytic capacity within the pool of screened participants (Spearman's r = 0.6, p = 0.03).

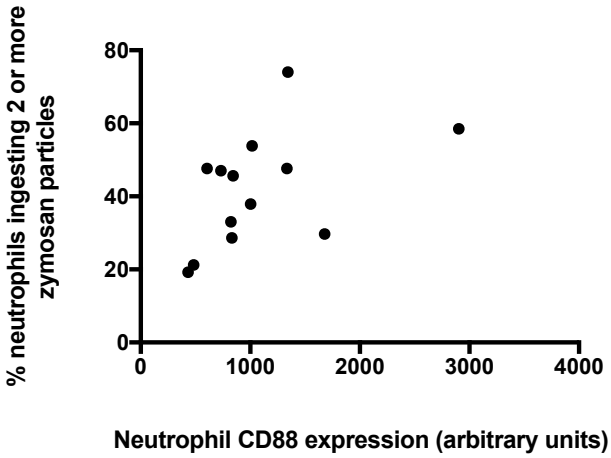

Figure 4l. Correlation between baseline neutrophil CD88 expression and neutrophil phagocytic capacity. (Spearman's r = 0.6, n= 13).

There remained a moderate correlation between neutrophil CD88 expression and neutrophil phagocytic capacity following the administration of GM-CSF, (r=0.43).

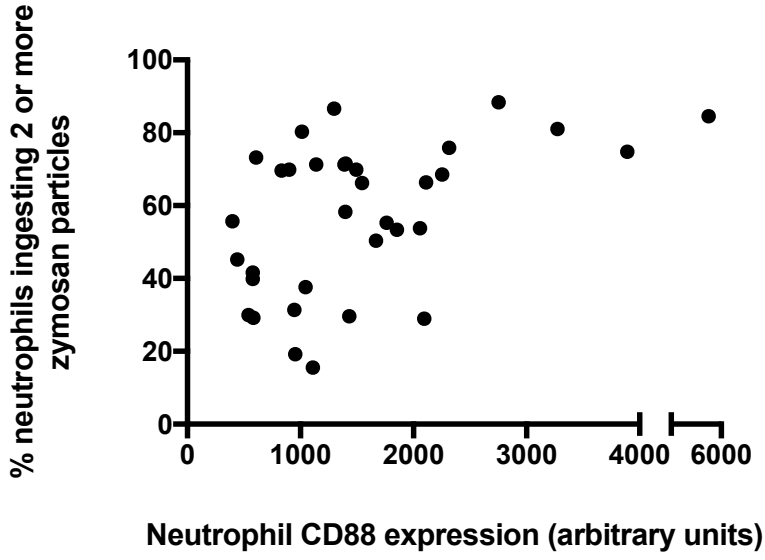

Figure 4m. Correlation between neutrophil CD88 expression and neutrophil phagocytic capacity following GM-CSF administration. (Spearman's r = 0.43, n= 34)

4.14 Serum GM-CSF concentration

Blood was collected each morning for measurement of serum GM-CSF concentration. In addition, samples were taken 2 hours, 4 hours and 6 hours post administration of GM-CSF on day 0 and day 3. Following collection these blood samples were kept at 4°C and transported to the lab immediately following collection of the 6-hour post-dose sample. ELISA failed in one of three patients in whom it was performed.

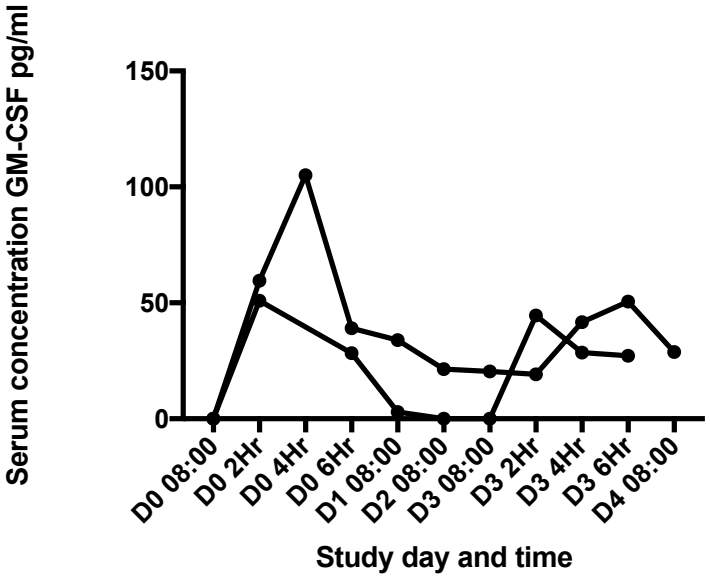

Figure 4n. Serum GM-CSF concentration following administration of subcutaneous rhu GM-CSF 3µg/kg. Serum GM-CSF levels measured by ELISA. (n=2)

4.15 PaO2:FiO2

As discussed, due to the concerns regarding the potential for GM-CSF to induce or exacerbate ALI/ ARDS PaO_2:FiO_2 was measured in all patients on a daily basis at baseline and up to day 9. This was included as part of usual clinical care. The maximum PaO_2:FiO_2 (kPa) was recorded for each 24-hour period. This assessment only took place when the patient had an arterial line in situ. Once an arterial line had been removed daily oxygen saturations and inspired oxygen concentration were recorded.

There was no evidence to suggest a deterioration in gas exchange, as measured by PaO2:FiO2, following administration of GM-CSF.

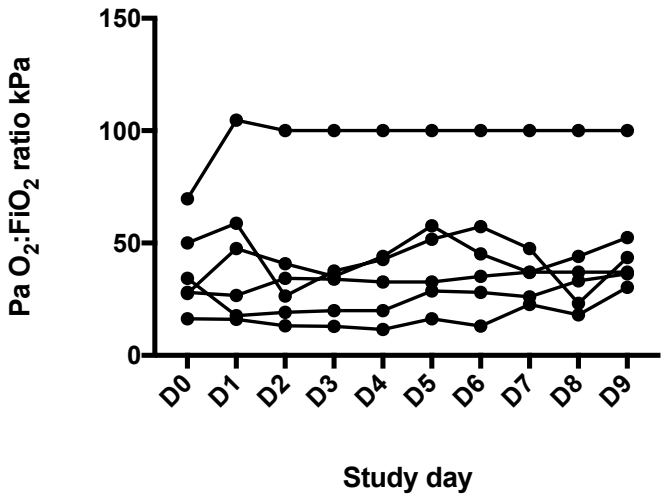

Figure 4o. Individual maximum daily PaO2:FiO2 over study period. Highest PaO2:FiO2 recorded each day for last 24 hours (kPa).

4.16 Adverse Events

4.16.1 Serious adverse events

As outlined in chapter 2 (section 2.13.3), because the study was recruiting patients with critical illness it was expected that many of them may experience serious adverse events as part of their disease course. It was therefore agreed prior to the study that such events should not be reported as SAEs. All other SAEs were to be reported. There were, however no SAEs reported during completion of the DFS.

4.16.2 Expected adverse events

Expected adverse events were recorded on a daily basis within the case report form (CRF). All patients experienced temperatures up to 38°C during the course of the study. It was not possible to ascertain whether these temperatures were due to the study drug or the patients' underlying conditions as in all cases patients were experiencing temperatures prior to commencing GM-CSF and the underlying conditions are frequently associated with elevated body temperature.

Expected adverse event	**Occurrence**
Temperature up to 38 C	6/6
Elevated white cell count	6/6
Reaction at injection site	0/6
Bone pain	0/6
Myalgia	0/6
'First dose syndrome'	0/6

Table 17. Expected adverse events

All patients had some degree of rise in their white cell count (WCC). This was extremely variable with the peak rise ranging from 3.2 to 28.3 $x10^9$/L. The general

trend was for the white cell count to rise over days 0-3 while the patient was receiving the study drug and then to fall slowly. There were no incidences of the white cell count rising above 50 x10^9/L (the cut off at which the protocol recommends stopping the drug or reducing the dose by half).

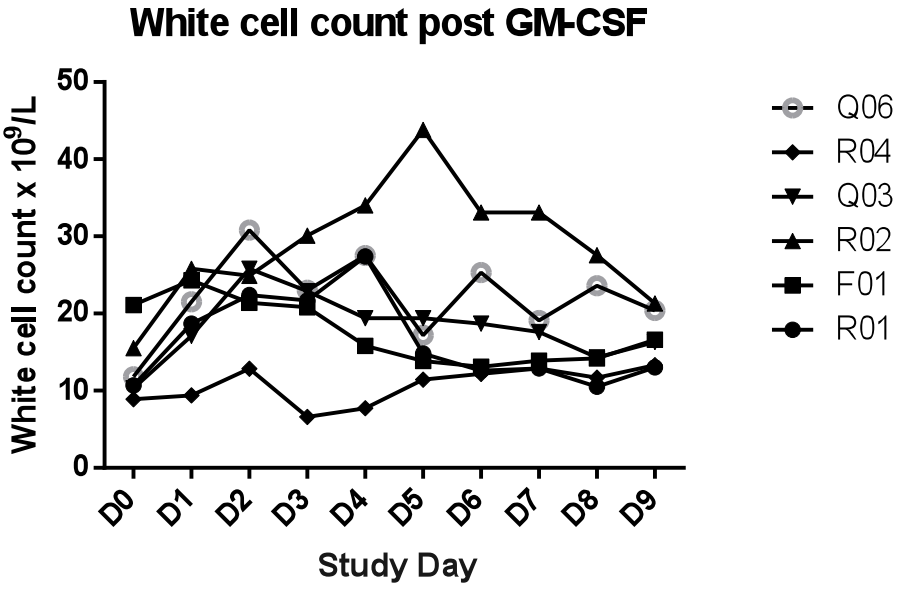

Figure 4p. Daily assessment of white blood cell count post GM-CSF

There were no recorded instances of a reaction at the injection site. No patients reported bone pain or myalgia. However, 5 out of 6 patients were intubated and sedated and therefore this may have been masked and was difficult to measure. No patients displayed signs of the first dose syndrome.

4.16.3 Unexpected adverse events

There were 3 reported adverse events documented in the adverse event pages of the CRF. These all occurred in the same patient (Q03). Two of these events were classified as being unlikely to be related to the study drug and one as possibly being related to the study drug. One event was deemed to be moderate in severity with the other two being deemed to be of mild severity. The patient concerned had been admitted with sepsis secondary to bacterial endocarditis. They had a background history of chronic liver disease secondary to alcohol excess, hypertension, chronic kidney disease and previous breast cancer. The patient was transferred to the regional cardio-thoracic intensive care unit on day 4 and underwent emergency aortic valve repair (AVR) the following day.

Nature of adverse event	**Severity**	**Relationship to drug**
Atrial Tachycardia (Q03)	Moderate	Unlikely - Occurred 2 hours post GM-CSF injection, patient agitated and fighting ventilator at time of onset
Hypotension (Q03)	Mild	Unlikely – Occurred 1 hour post GM-CSF injection, patient had just been put back onto continuous veno-venous haemofiltration (CVVH)
Thrombocytopaenia (Q03)	Moderate	Possibly - Patient was septic, history of chronic liver disease, on CVVH. Other causes deemed more likely

Table 18. Unexpected adverse events

4.17 Safety bloods

Bloods were taken each day to look for unwanted side effects of GM-CSF which may have compromised patient safety. Daily analysis of full blood count, urea and electrolytes and liver function tests was performed. GM-CSF is known to increase the number of circulating cells in the myeloid population. There have been reports of rises in bilirubin, hepatic enzymes and creatinine being seen in some patients with pre-existing renal and hepatic disease within uncontrolled trials.

4.17.1 Neutrophil count

All patients had some degree of rise in their neutrophil count post administration of GM-CSF. The response was very variable and probably also reflected individual changes in clinical condition. The maximum rise in neutrophil count was to a peak of 35.5 $x10^9$/L.

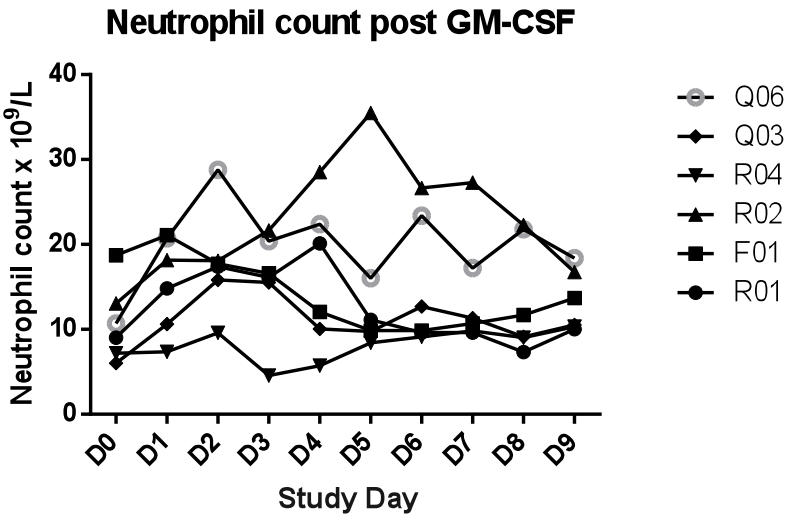

Figure 4q. Daily assessment of neutrophil count post-GM-CSF

4.17.2 Platelet count

One patient experienced a fall in their platelet count while receiving the study drug. The fall in platelet count was thought to be possibly related to the drug by the patient's clinical team. However other causes were felt to be more likely. The patient had a diagnosis of bacterial endocarditis and a background history of chronic liver disease and was on CVVH at the time. In view of the fall in platelet count the final dose of GM-CSF was not administered on day 3. Other patients tended to have a rise in their platelet count following administration of the study drug.

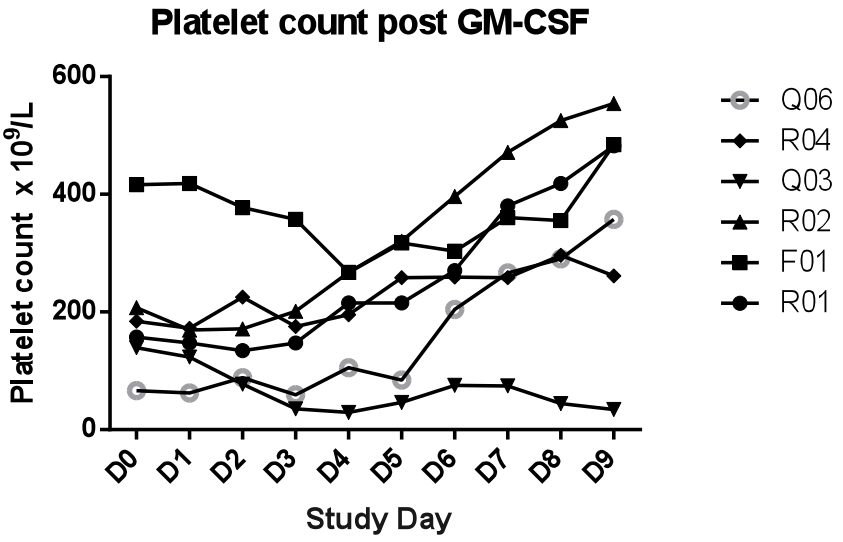

Figure 4r: Individual platelet count data over study days 0-9

4.17.4 Creatinine

There were no obvious changes seen in creatinine level post GM-CSF. Two patients (Q03 and Q06) were receiving CVVH during the study period patient.

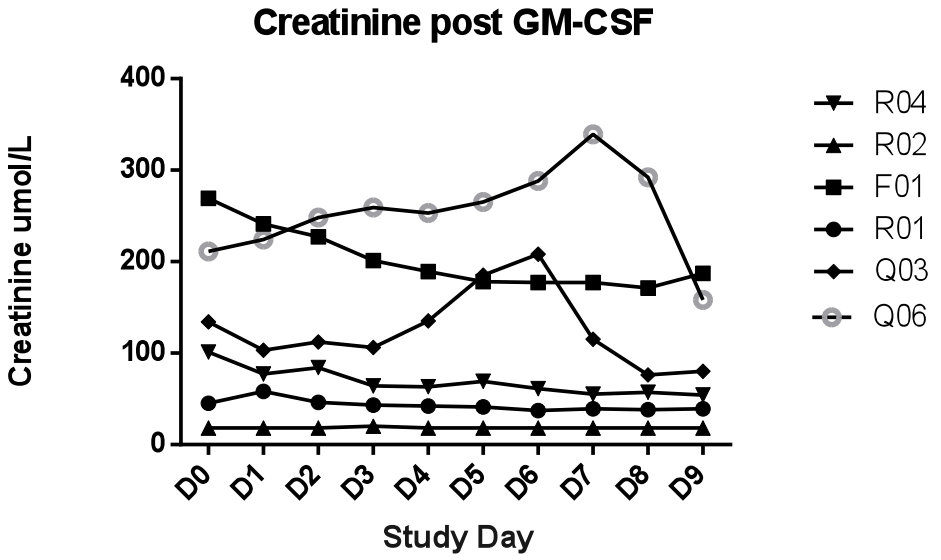

Figure 4s: Creatinine data over study days 0-9

4.17.5 Liver function tests

In this study elevation in liver enzymes was seen in 2 patients. The first patient R04 was admitted with a perforated oesophagus and had elevated aspartate aminotransferase (AST) on admission. He commenced GM-CSF 2 days following admission to ICU. His perforation was not endoscopically apparent initially and he remained septic with bilateral pleural empyema for several days. He was subsequently commenced on total parenteral nutrition (TPN). His alanine transaminase (ALT) and alkaline phosphatase (ALP) rose steeply from study day 3 peaking on study day 9. His LFTs settled gradually over several weeks following a second peak in ALP/ALT 3 weeks following admission.

In the second patient, R01, this occurred 10 days after the drug had been stopped (not illustrated) and coincided with an increase in WCC, C reactive protein (CRP) and temperature in association with right upper quadrant pain. An ultrasound scan at the time showed the presence of gravel-like calculi in the gallbladder. The rise and fall in liver enzymes occurred quickly and the patient was followed up until complete

resolution of their liver function abnormality had occurred. These findings were discussed in a DMSC meeting and thought to be unlikely to be related to the study drug.

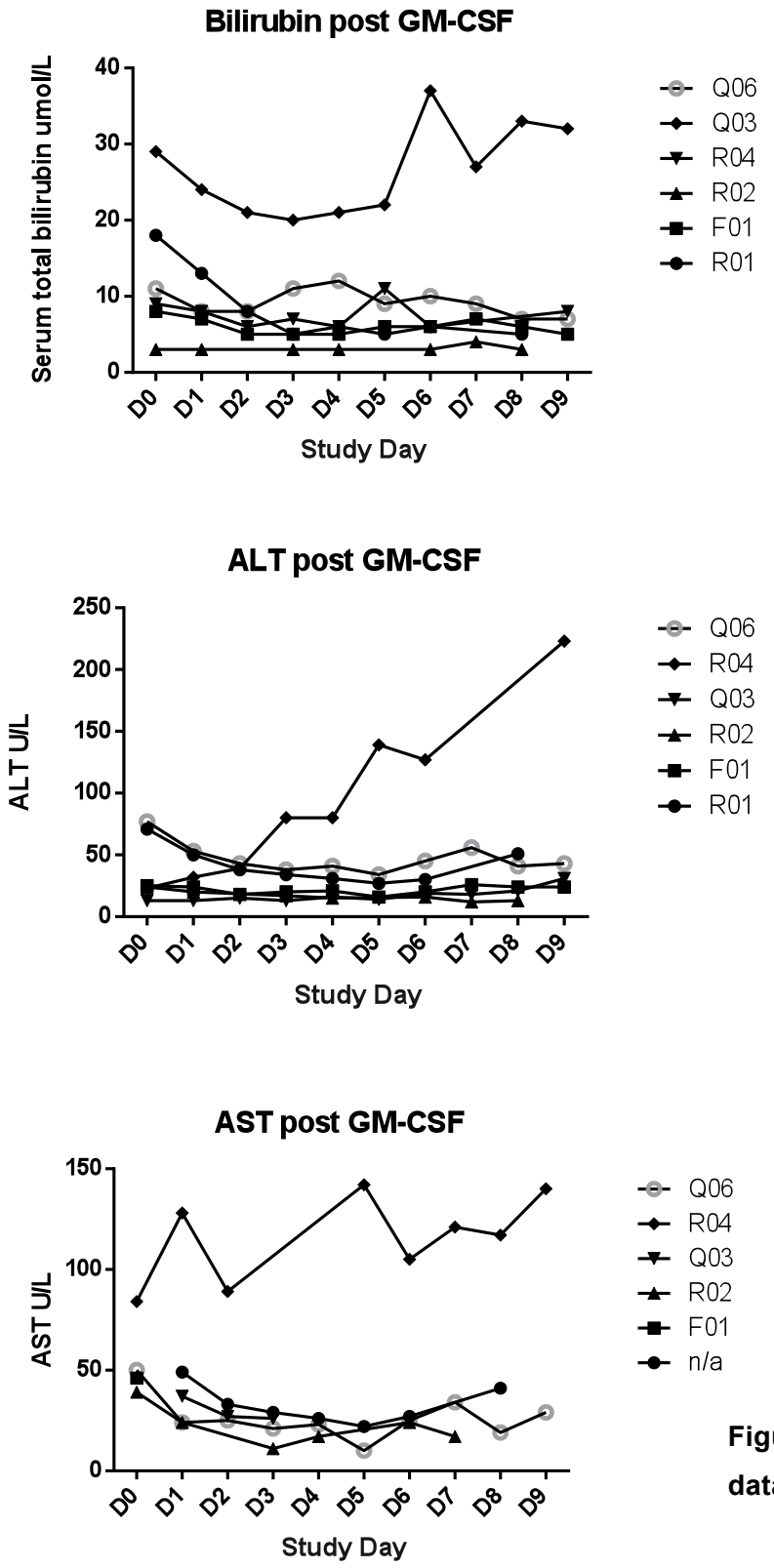

Figure 4t: Individual liver function test data over study days 0-9

4.18 Outcome

At 30 days all six patients were alive. Two patients had been discharged home from hospital, 1 had been discharged from ICU but remained a hospital inpatient, 3 patients remained on ICU.

One patient subsequently died (Q03) 2 months post GM-CSF treatment. This patient was admitted with bacterial endocarditis and was transferred to the local cardio-thoracic unit for emergency AVR. They had a slow wean from the ventilator post operatively with the patient finally being weaned off all ventilatory support after approximately 7 weeks. Shortly afterwards the patient deteriorated with a further episode of sepsis and after discussion with the family a decision was made not to re-escalate care due to her pre-existing frailty and poor prognosis.

Study Number	30 day outcome	Final outcome
R01	Discharged home	Discharged home day 16
F01	Surgical inpatient	Discharged home at 3 months
R02	HDU inpatient	Medical inpatient at 5 months
Q03	ITU inpatient	Died 2 months post GM-CSF
R04	Discharged home	Discharged home day 30
Q06	ITU inpatient	Discharged home at 4 months

Table 19: Outcome for patients recruited to dose-finding study

4.19 Discussion

The DFS set out to determine the optimum dose and duration of GM-CSF that would both be effective in terms of producing a demonstrable increase in neutrophil phagocytic capacity, and demonstrate an acceptable safety profile. In terms of safety, it was pre-determined that the dose which would be carried forward to the RCT should be associated with dose-limiting toxicity in less than 2 of the 6 patients within the cohort in which it had been demonstrated to be effective (Trial Protocol Section 5.6.1.3, appendix).

During the conduct of the DFS, prior to analysis of the data, it was also determined that if low-dose and short duration GM-CSF was found to be both safe and effective i.e. resulting in neutrophil phagocytic capacity which would be protective against ICUAI (≥ 50%) then the trial may proceed directly to RCT without dose escalation (Trial Protocol, appendix).

4.19.1 Optimum dose and duration of GM-CSF

4.19.1.1 Effect of GM-CSF on neutrophil phagocytic capacity

GM-CSF administered at a dose of 3μg/kg/day for a duration of 4 days was seen to be associated with an increase in neutrophil phagocytic capacity in all patients. On day 2, the day for assessment of the primary outcome, neutrophil phagocytic capacity was seen to be above 50% in all patients (apart from the one patient whose phagocytic capacity assessment failed on this day and therefore could not be measured). Neutrophil phagocytic capacity ≥50% has previously been shown to be the level associated with less ICU acquired infection (Conway Morris et al 2011). The difference in mean neutrophil phagocytic capacity between day 0 and day 2 was 20.9%. This is in excess of the absolute mean difference in neutrophil phagocytic capacity, applied to our power calculation for the sample size required to detect a difference between groups for the RCT with a power of 90% (chapter 2, section 2.18.3). The mean rise, on day 2, in individual neutrophil phagocytic was 22.7%. The response to GM-CSF varied however with the minimum rise, on day 2, being 8.3% leading to a mean phagocytic capacity of 55.3% for the patient concerned.

In all but one patient a continued improvement in neutrophil phagocytic capacity was seen at day 7 which was the last day on which all 6 patients were sampled (due to samples not always being collected on weekend days when no researcher was

available). Mean neutrophil phagocytic capacity on day 7 was 65.9% (s.d. 19.2) compared with day 2 mean of 59.6 (s.d. 7.1) and day 0 mean 38.7 (s.d.10.6). This may suggest that the effect of GM-CSF on neutrophil phagocytic capacity persisted after the treatment had been discontinued on day 3. This difference did not reach statistical significance however due to the decline in neutrophil phagocytic capacity in one patient (Q03) on this day.

Patient Q03 had been admitted with sepsis secondary to bacterial endocarditis on a background of chronic liver disease. As a result of the bacterial endocarditis affecting the aortic valve the patient became increasingly unstable necessitating transfer to a regional cardiothoracic unit and was required to undergo emergency cardiothoracic surgery to replace the aortic valve on day 5 of their admission. The cardiothoracic surgery required the patient to be on cardiopulmonary bypass. Cardiopulmonary bypass has previously been shown to impair neutrophil phagocytosis for at least 12 hours following surgery (Hamano et al., 1996) and this may well have accounted for the decline in this patient's neutrophil phagocytic capacity following an initial apparent response after the administration of GM-CSF.

Interestingly all patients were seen to have an initial fall in their neutrophil phagocytic capacity on day 1 post administration of GM-CSF (mean neutrophil phagocytic capacity day 1 = 31.6% (s.d. +/- 8.8) vs day 0 = 38.7% (s.d +/- 10.6). As GM-CSF is known to result in an increase in the absolute number of circulating neutrophils it may be that the effects in terms of neutrophil number occur more quickly than the effects in terms of neutrophil phagocytic activity therefore resulting in a net reduction in neutrophil phagocytic capacity on day 1 followed by a net rise by day 2.

4.19.1.2 Safety profile of GM-CSF

The administration of GM-CSF was not associated with a serious adverse event in any of the patients treated in the dose finding study. The occurrence of common adverse events was recorded on a daily basis in the case report form, namely; rise in white cell count, the development of a temperature up to 38°C, a reaction at the injection site, bone pain, myalgia and the first dose syndrome (Steward, 1993). All patients were seen to have a rise in their white cell count and in their body temperature following administration of GM-CSF. In no cases was there a rise in the total white cell count to a level which would trigger the study drug termination criteria

or be associated with the potential of a complication related to leucocytosis. As documented previously it was not possible to determine whether the rises seen in white cell count and body temperature were due to the patients underlying clinical condition or the effects of GM-CSF. Both indices, while known side effects of GM-CSF therapy, are frequently elevated in critical illness and form part of the SIRS criteria used to establish eligibility for participation in the trial (Members of the ACCP/SCCM Consensus Conference Committee, 1992).

There were no instances of a reaction at the injection site in any patient. Neither bone pain nor myalgia were reported however 5 of the 6 patients were sedated and ventilated and therefore detection of this was extremely difficult. Finally, the 'first dose response' which manifests as hypotension, dyspnea and flushing during or shortly after administration of the first dose only, was not observed in any of the 6 participants.

All three reported adverse events occurred in the same patient. Two of these events were assessed as being unlikely to be related to the study drug. The one adverse event which was recorded as being possibly related to GM-CSF, the development of thrombocytopaenia, was deemed to be of moderate severity. GM-CSF has been more commonly associated with the development of thrombocytosis (Vesole et al 1994, Sanofi-Aventis, 2013). Increased levels of GM-CSF have however also been observed in patients with both immune and non-immune thrombocytopaenia (Abboud et al., 1996). The patient in whom this adverse event was reported had a history of chronic liver disease secondary to alcohol excess and was receiving CVVH for acute renal failure, both of which can be associated with the development of thrombocytopaenia. In this case, it was felt that the observed thrombocytopaenia was more likely to have been a result of these factors. To ensure patient safety, however, and to avoid the potential for a further decline in platelet count, which could potentially lead to a risk of bleeding, the final dose of GM-CSF was withheld from this patient. All other patients tended to show a degree of rise in their platelet count following administration of GM-CSF.

There were no other instances of an alteration in blood profile leading to triggering of the study drug termination criteria. As expected GM-CSF administration was associated with a rise in both total white cell count and neutrophil count in all recipients (Sanofi-Aventis, 2013), however, the response was quite variable and the

counts were seen to decline slowly on completion of the study drug dosing schedule on day 3.

The Leukine Summary of Product Characteristics reports that in some patients with pre-existing renal or hepatic impairment alterations in these blood markers of renal or hepatic dysfunction have been observed in uncontrolled trials. No statistically significant difference has been demonstrated in controlled trials (Sanofi-Aventis, 2013). In terms of renal function there was no significant rise in creatinine seen in any of the patients participating in the dose finding study. Two patients were receiving CVVH prior to and during the treatment period. Alteration in liver function was however observed in 2 patients during the study treatment and follow-up period. In neither case did this lead to a reduction in or cessation of the study drug. In one patient, R04, this occurred on study day 3 and resolved slowly over several weeks. In the second patient the rise in liver function occurred 10 days after completion of the study drug and was thought to be unlikely to be related by the independent DMSC.

4.19.1.3 Selection of dose of GM-CSF for use in RCT

Low dose (3μg/kg/day), short duration (4 days) subcutaneous GM-CSF was seen to be both effective (associated with a demonstrable increase in neutrophil phagocytic capacity, in all patients, to a level associated with protection against ICUAI) and safe (producing a dose-limiting toxicity in < 2 of the 6 patients in whom it had been shown to be effective) in the first cohort of patients who were recruited to the DFS. On the basis that an escalation in dose may be associated with an increase in adverse events but was unlikely to confer additional clinical benefit a decision was made to proceed directly to the RCT at this dose, in keeping with the study protocol.

4.19.2 Study conduct

As discussed, because of the concerns regarding the potential for a serious adverse reaction to the administration of GM-CSF, in particular in relation to the possibility of inducing ALI/ARDS (Goodman et al., 1999), the DMSC directed that patients should be recruited 1 at a time for the first 3 patients with submission of a detailed report to the DMSC prior to recruitment of the subsequent patient. Whilst this was a necessary requirement, to ensure patient safety as a priority, it had a significant impact on recruitment to the study. The DFS had initially been estimated to be completed within approximately six months with a total of 24 patients being recruited to 4 different dosing schedules. In reality, it took a total of seven months to recruit 6 patients to the first sub-group of the dose finding study and this undoubtedly had an influence on the decision to proceed directly to RCT with low-dose short-duration GM-CSF when an effect was seen at this dose. Again, as was seen in the ACS there was a very high screening to eligibility ratio and subsequently a 4:1 ratio of patients meeting the initial eligibility criteria to those who went on to receive the study drug.

4.19.3 Suitability of inclusion / exclusion criteria

The dose finding study offered the opportunity for further assessment and analysis of the suitability of the inclusion and exclusion criteria set for entry into the RCT. It was noted that despite the changes made prior to the DFS there remained 3 particular groups of patients within the target population who may be being excluded unnecessarily.

I. Patients with a history of cancer (unless curative resection or treatment performed)
II. Critically ill patients with an initial requirement for organ support but whose need for organ support had resolved prior to administration of the first dose of GM-CSF
III. Patients admitted to ICU outside of the 48-hour window necessary for inclusion

Colony stimulating factors have been widely used for many years in patients undergoing chemotherapy for solid organ malignancies to aid recovery of neutrophil numbers. More recently however there have been concerns in relation to the potential for GM-CSF to act as a stimulant to tumour growth in patients with GM-CSF receptor positive tumours and that some of these tumours may follow a particularly aggressive pathway with accelerated progression (Aliper et al., 2014). Meanwhile, however, GM-CSF has been investigated as an adjuvant therapy in the treatment of certain solid organ malignancies including prostate cancer and melanoma (Amato et al., 2008, Grotz et al., 2014) with evidence of tumour response and delayed recurrence.

During the screening process we encountered numerous patients with solid organ malignancies, many of whom had very indolent disease including low grade prostate cancer and a history of previous breast and bladder cancer under long-term follow-up without evidence of recurrence but in whom cure could not be guaranteed. Our concern was that many of these critically ill patients could potentially stand to benefit from treatment with GM-CSF during critical illness but were being excluded from the trial on the basis of their history of malignancy. In addition, these patients represent a significant proportion of the ICU population. We sought advice from two independent oncologists regarding the need to exclude these patients from our trial. Both were of the opinion that the potential of benefit to such patients from participation in the trial far outweighed the risk of stimulation of an underlying malignancy by GM-CSF. Consequently, following discussion with the DMSC an application was made to amend the exclusion criteria by removal of this point and this was approved.

During the DFS a total of 3 patients had to be excluded from the trial following the consent process due to a resolution of their need for organ support prior to the first administration of GM-CSF. This represented almost 18% (3/17) of the consented patients. It was observed that while these patients no longer had a need for organ support they remained critically ill. In the 2 patients whose organ support was discontinued after blood had been drawn for the final assessment of eligibility the neutrophil phagocytic capacity was found to be 19.2% and 28.6% putting them both at significant risk for the development of an ICUAI. It was acknowledged by all within both the trial and clinical teams that cessation of organ support did not indicate a reduction in risk of ICUAI nor a recovery in terms of neutrophil phagocytic capacity

and that excluding these patients at this stage, on these grounds was inappropriate. An additional amendment was therefore sought to alter this inclusion criterion to read

- Has required support of one or more organ systems (invasive ventilation, inotropes or haemofiltration) during current ICU stay

This amendment was approved by the relevant regulatory authorities and supported by the independent DMSC.

Finally, as screening did not routinely take place on a Saturday and Sunday, when no researcher was available, it became apparent that patients admitted between Friday lunchtime (after screening had taken place) and early Saturday morning (more than 48 hours before screening took place on a Monday morning) were consequently being excluded from the study by virtue of the time of their admission to ICU. It was felt to be inappropriate to be excluding a cohort of patients on this basis and therefore a final application was made to extend the inclusion criteria for patients to 72 hours following their admission to ICU. This amendment was again approved and put in place prior to proceeding to the RCT.

4.19.4 Consent issues

It was noted that there was a higher incidence of declined consent by PerLRs and ProfLRs in the DFS than was observed in the ACS. This was perhaps not unexpected in view of the nature of the intervention in the DFS. Although the sample size did not allow for any statistical comparative analysis it was noted by the researchers during the process of seeking informed consent that PerLRs were more likely to decline consent on behalf of an older patient often saying that they felt that their relative had been through enough and had less to gain from participation in a clinical trial at their age. This may account for the slight difference seen in the median age of the cohort in the DFS compared to the ACS (DFS median age 56.5 years (range 25-66) versus ACS median age 66yrs (range 31-85)).

4.19.5 Effect of GM-CSF on monocyte HLA-DR expression

As in the ACS monocyte HLA-DR was seen to be low in the majority of patients with 5 of the 6 patients having an mHLA-DR level below 15,000 Ab/cell indicative of moderate to severe immunodepression. Median mHLA-DR was very similar in the patient cohort in the DFS compared to the ACS with median of 4297 Ab/cell compared to 4796 Ab/cell respectively.

Monocyte HLA-DR was seen to rise following the administration of GM-CSF until day 3-4 following which it was seen to fall again. Levels rose to above 20,000 Ab/cell in 4 of the 6 patients suggestive of restoration of immune competence. The subsequent fall following completion of GM-CSF may suggest that the effect of GM-CSF on the restoration of immune function was lost quickly on cessation of the study drug on day 3. In a similar study by Meisel et al looking at the effect of GM-CSF on monocyte de-activation (Meisel et al., 2009), GM-CSF was continued in all patients for 8 days. At day 5 all patients with a mHLA-DR level below 15,000 Ab/cell had their dose increased from 4μg/kg/day to 8μg/kg/day. All 19 patients in this study cohort achieved a monocyte HLA-DR level > 15,000 Ab/cell which they classified as normal (Meisel et al., 2009). The response to GM-CSF in Meisel's study was greater than that seen in our patient cohort with mean mHLA-DR 43,676 (+/-24,517) at day 4 versus 5,609 (+/- 3,628) at baseline in Meisel's study compared to mean mHLA-DR 14,279 (+/- 3243) on day 4 versus 7,814 (+/- 7343) at baseline in our own cohort. In Meisel's study all patients received a 33% greater dose of GM-CSF subcutaneously each day.

While mHLA-DR is widely accepted as a reliable marker of immune dysfunction the biological significance of the restoration of circulating MHLA-DR expression following treatment with GM-CSF is not clear. No assessment of the immune function of monocytes following recovery of mHLA-DR expression has been reported in the literature to my knowledge. Within our patient cohort despite a weak correlation between mHLA-DR and neutrophil phagocytic capacity at baseline no correlation was seen between mHLA-DR and neutrophil phagocytic capacity following administration of GM-CSF.

The significance of the fall in mHLA-DR following completion of the GM-CSF dosing schedule is not clear but may potentially indicate an inadequate duration of treatment to ensure maintenance of restoration of immune function.

4.19.6 Effect of GM-CSF on neutrophil CD88 expression

Within this cohort of patients there was a general trend towards an increase in neutrophil CD88 expression following the administration of GM-CSF. As had been reported previously by Conway Morris et al there was a significant correlation seen between neutrophil CD88 expression and neutrophil phagocytic capacity at baseline (Spearman r = 0.6). A moderate correlation was seen to persist following administration of GM-CSF (Spearman r = 0.43). In Conway Morris' previous ex-vivo work the correlation between neutrophil CD88 expression and neutrophil phagocytic capacity was not studied following the application of GM-CSF. GM-CSF was seen to restore phagocytic capacity but the effect on restoration of neutrophil CD88 expression was not assessed (Conway Morris et al., 2009).

4.19.7 Serum GM-CSF levels following administration of study drug

Serum GM-CSF levels were seen to rise following subcutaneous injection of GM-CSF peaking at between 4 and 6 hours post-dose. The pharmacokinetics of GM-CSF are variably reported with studies showing a range in time to peak serum GM-CSF levels following administration of a bolus sub-cutaneous injection between 1 – 20 hours post dose (Steward,1993, Hovgaard et al., 1991, Cebon et al., 1988). Measured peak serum GM-CSF levels in our cohort were between 50-110 pg/ml. Similar levels were seen in Meisel's study where the treated cohort mean GM-CSF levels were 40.3 (+/-77.1) pg/ml. Pharmacokinetic studies of GM-CSF have shown peak serum levels to be related to both the dose and route of administration of GM-CSF. The sub-cutaneous route of administration was chosen for use in this study due to its more favourable safety profile (Honkoop et al., 1996). There are limited data on the absorption of drugs administered subcutaneously in critically ill patients however available studies suggest that absorption may be significantly impaired due to poor tissue perfusion particularly associated with vasopressor use (Smith et al., 2012, Dorffler-Melly et al., 2002). One study examining serum anti-factor Xa levels in critically ill patients on ICU receiving low molecular weight heparin (the most commonly administered sub-cutaneous drug in ICU) showed that only 28% of patients receiving the recommended dose were found to have reached the desired range for thromboprophylaxis (Jochberger et al.,2005). Multiple factors affect the bioavailability of drugs in critically ill patients on ICU and careful pharmacokinetic

studies are needed to determine effective doses for such patients as data from non-critically ill cohorts are unlikely to be representative.

4.19.8 PaO_2:FiO_2 following administration of GM-CSF

The administration of GM-CSF was not associated with any deterioration in gas exchange as measured by the PaO_2:FiO_2 and therefore there was no evidence to suggest the development or worsening of acute lung injury in these patients. 4 of the 6 patients met the PaO_2:FiO_2 criteria for ALI on day 0, prior to administration of GM-CSF, with 2 patients meeting the criteria for ARDS (Bernard et al.,1994). Median PaO_2:FiO_2 was 31kPa (range 16-70). There was a general trend towards an improvement in PaO_2:FiO_2 over the course of the study but this did not meet statistical significance. By day 9 median PaO_2:FiO_2 had risen to 40kPa (range 30-100) with 3 patients meeting the criteria for ALI and no patients having ARDS. The concerns regarding the sequestration of neutrophils within the lungs leading to ALI were not apparent in this cohort of patients (Sanofi-Aventis., 2013). The data regarding the role of GM-CSF in the development of ALI in critically ill patients is conflicting with one recent randomised controlled trial investigating whether GM-CSF may in fact be useful as a therapy to treat patients with ARDS. There was no difference seen in the primary outcome of ventilator free days seen (Paine et al., 2012).

4.19.8 Outcome of patients following administration of GM-CSF

5 of the 6 patients survived to 30 days following administration of GM-CSF; 2 had been discharged home, one remained an inpatient on ICU and 2 remained hospital inpatients not on ICU. 1 patient was lost to follow-up at 30 days. As with the ACS this represents an expected outcome for critically ill patients.

4.20 Limitations of the dose finding study

The dose finding study had several limitations which may have impacted on the interpretation of the results.

4.20.1 Sample size

The small sample size limits the strength of any findings and in particular lead to a significant impact of the missing phagocytosis data for patient R01 on day 2. A larger cohort of patients in each dosing sub-group would have offered greater confidence in the observed biological effect.

4.20.2 Lack of control group

A control group within each dosing cohort to run in parallel to the treatment group would have enabled analysis of the natural course of neutrophil phagocytic capacity during critical illness. As we did not have a control group for comparison and the literature lacks a description of the natural recovery of neutrophil phagocytic capacity in the course of critical illness, we were unable to assess the magnitude of the apparent biological response which we observed in our cohort of treated patients. It is possible therefore that we may have overestimated the contribution of the GM-CSF to the improvements seen in individual neutrophil phagocytic capacity.

4.20.3 Absence of comparison with increased dose and duration of GM-CSF

The decision to proceed directly to the RCT carrying forward low-dose, short-duration GM-CSF as the treatment intervention was made partly on the basis that low-dose, short-duration GM-CSF was deemed to be both safe and effective and partly due to pressures in relation to recruitment to, and progress of, the trial. Proceeding to the next stages of the DFS would have enabled comparison of both the increased dose (in particular, whether an increased biological effect may have been observed), and the more prolonged duration which may have shown evidence of a more sustained response in terms of mHLA-DR recovery suggesting a potentially increased benefit with a longer duration of therapy.

4.21 Conclusions

The dose finding study demonstrated low-dose, short duration GM-CSF to be both safe and effective in terms of restoring neutrophil phagocytic capacity in critically ill patients with confirmed impairment. The absence of any deterioration in gas exchange confirmed that the RCT could safely proceed with open, unrestricted recruitment of patients without fear of harm. Finally, the opportunity to further analyse the inclusion and exclusion criteria enabled these criteria to be further modified to ensure recruitment of the target population of patients most likely to have the potential for benefit from the intervention being studied.

Chapter 5: Randomised Controlled Trial

This chapter will outline the results of the randomised controlled trial. It will begin by stating the aim of the study and describing the primary and secondary outcomes and will go onto give a synopsis of the study conduct. The processes of screening, recruitment and consent will be revised prior to presenting the results. Finally, the conclusions and limitations of the study will be discussed.

5.1 Aim of study

The aim of the trial was to investigate the hypothesis that GM-CSF would restore effective neutrophil function in critically ill patients with known impaired neutrophil function.

5.2 Study design

The randomised controlled trial was designed to compare the outcome, in terms of neutrophil phagocytic capacity, of 2 matched groups of critically ill patients with impaired neutrophil function randomised to receive either subcutaneous GM-CSF or placebo over a period of 4 days following admission to ICU. Based on the previous work carried out by our collaborators (Conway Morris et al, 2009) we believed that an absolute increase in neutrophil phagocytic capacity of ≥ 15% from baseline to day 2 would be clinically significant. On this basis we calculated that a sample size of 17 per group would give a power of 90% to detect such a difference between the groups. To allow for loss of patients to follow-up during the study we planned to recruit 19 patients into each group (Trial protocol, appendix).

Following completion of the Dose Finding Study it was recognised that some of the exclusion criteria were excluding patients within the target study population. As discussed in chapter 4 minor modifications to the inclusion and exclusion criteria were made and approved by the relevant regulatory bodies prior to commencing enrolment to the dose finding study;

I. The exclusion criterion ‘Patients with a history of cancer (unless curative resection or treatment performed)’ was removed
II. The window for inclusion following admission to ICU was extended to 72 hours
III. The inclusion requirement for organ support was amended to include any patient who had ‘required support of one or more organ systems (invasive ventilation, inotropes or haemofiltration) during current ICU stay’

5.3 Study outcomes

5.3.1 Primary outcome

The primary outcome of the trial was neutrophil phagocytic capacity, as measured by the percentage of neutrophils ingesting 2 or more zymosan particles at day 2 following administration of the study drug or placebo.

5.3.2 Secondary outcomes

The study included several secondary outcomes which included both biological and clinical measures.

5.3.2.1 Biological measures

The following biological measures were assessed up to day 9:

I. Sequential neutrophil phagocytic capacity to look for sustainability of any effect seen on day 2 following administration of GM-CSF
II. Neutrophil superoxide generation following administration of GM-CSF
III. Neutrophil chemotaxis following administration of GM-CSF
IV. Neutrophil apoptosis following administration of GM-CSF
V. Monocyte HLA-DR expression following administration of GM-CSF
VI. Relative proportions of regulatory T cells following administration of GM-CS

5.3.2.2 Clinical measures

I. Sequential organ failure assessment (SOFA) score to day 7
II. Length of ICU and hospital stay
III. The incidence of nosocomial infection (as defined by the HELICS criteria)
IV. All-cause mortality at 30 days following randomisation
V. PaO_2:FiO_2 following administration of GM-CSF to day 9
VI. Duration of mechanical ventilation to day 30

5.4 Study conduct

Following completion of the DFS (including analysis of the data collected in relation to the effect of GM-CSF on PaO_2:FiO_2) the DMSC gave permission for recruitment to the RCT to take place in an unrestricted manner with recruitment commencing at all sites at the same time.

5.5 Recruitment

The randomised controlled trial commenced in November 2013 and ran until March 2014. Patients were recruited from 5 different intensive care units across 3 different hospital Trusts in the North East of England namely, the Royal Victoria Infirmary (General and Neurosurgical ICUs) and the Freeman Hospital Critical Care Unit (all The Newcastle upon Tyne Hospitals NHS Foundation Trust), the Queen Elizabeth Hospital Gateshead Critical Care Unit, (Gateshead Health NHS Foundation Trust), and Sunderland Royal Hospital Integrated Critical Care Unit, City Hospitals Sunderland NHS Foundation Trust.

All admissions to critical care were screened on a daily basis, from Monday to Friday, to identify eligible patients according to the inclusion and exclusion criteria below.

Eligibility criteria for RCT

Inclusion criteria

- Fulfil criteria for SIRS* (see Chapter 2, section 2.1.1.3, page 40)
- Has required support of 1 or more organ systems (mechanical ventilation/inotropes/haemofiltration) during current ICU stay
- Survival over next 48 hours most likely outcome
- Consent obtained from patient, PerLR or ProfLR
- Admitted to ICU within last 72 hours

Final step in eligibility

- Neutrophil phagocytosis < 50%

Exclusion Criteria

- Absence/refusal of informed consent
- Current prescription of a colony stimulating factor
- Any history of allergy/adverse reaction to GM-CSF
- Total white cell count >30x10^9/litre at time of screening
- Haemoglobin < 7.5g/dl at the time of screening
- Age < 18 years
- Pregnancy or lactation
- Known in-born errors of neutrophil metabolism
- Known haematological malignancy and/or known to have >10% peripheral blood blast cells
- Known aplastic anaemia or pancytopaenia
- Platelet count <50x10^9/litre
- Chemotherapy or radiotherapy within the last 24 hours
- Solid organ or bone marrow transplantation
- Use of maintenance immunosuppressive drugs other than maintenance corticosteroids (allowed up to 10mg prednisolone/day or equivalent)
- Known HIV infection
- Active connective tissue disease (e.g. rheumatoid disease, systemic lupus erythematosus) requiring active pharmacological treatment.
- ST-segment elevation myocardial infarction, acute pericarditis (by ECG criteria) or pulmonary embolism (radiographically confirmed) in previous week
- Involvement in any study involving an investigational medicinal product in the previous 30 days

Table 20: Eligibility criteria for randomised controlled trial

A total of 3634 patients were screened to recruit 38 patients to the RCT. The CONSORT (Consolidated standards of Reporting Trials) diagram (Figure 5.1) overleaf shows the process by which the 38 patients were enrolled into the study.

As with the previous phases of the study consent was sought from the individual where possible (8/38, 21.1%). Of these participants 30 (78.9%) lacked capacity as a consequence of their critical illness and in these circumstances, as before, consent was sought from a personal (26/38, 68.4%) or professional (4/38, 10.5%) legal representative. 18 of the 30 (60%) participants who lacked capacity at the time of enrolment recovered capacity prior to end end of the trial. Of these 13 (13/18, 72) gave retrospective consent. Of the other 5 one had been withdrawn from the study immediately following randomisation and received no drug or further blood sampling and 2 were discharged from hospital prior to retrospective consent being sought. There were substantially more cases of declined consent in the RCT with 70 episodes of out of 134 eligible patients (52%) compared to 7/24 (29%) in the DFS.

5.6 Final eligibility

As with the DFS neutrophil phagocytic capacity assessment was used as the final step for eligibility with only those patients found to have neutrophil phagocytic capacity < 50% proceeding to randomisation.

5.7 Randomisation

Once final eligibility was confirmed patients were randomised via an online programme and a randomisation number was generated. A prescription containing the randomisation number was then taken to the relevant clinical trials pharmacy where study drug/placebo was issued to a member of the unblinded research nursing team delegated with responsibility for administering the drug. Blinding of all other clinical and research staff was maintained during drug administration.

21 patients were randomised to receive placebo with 17 patients being randomised to receive GM-CSF (see consort diagram, figure 5.1). The reason for the imbalance between groups was in part due to the randomisation being stratified by site in permuted blocks of variable length (Trial protocol, appendix 1) and part due to the effect of one patient who was randomised in error and was subsequently excluded from the study.

5.8 CONSORT diagram

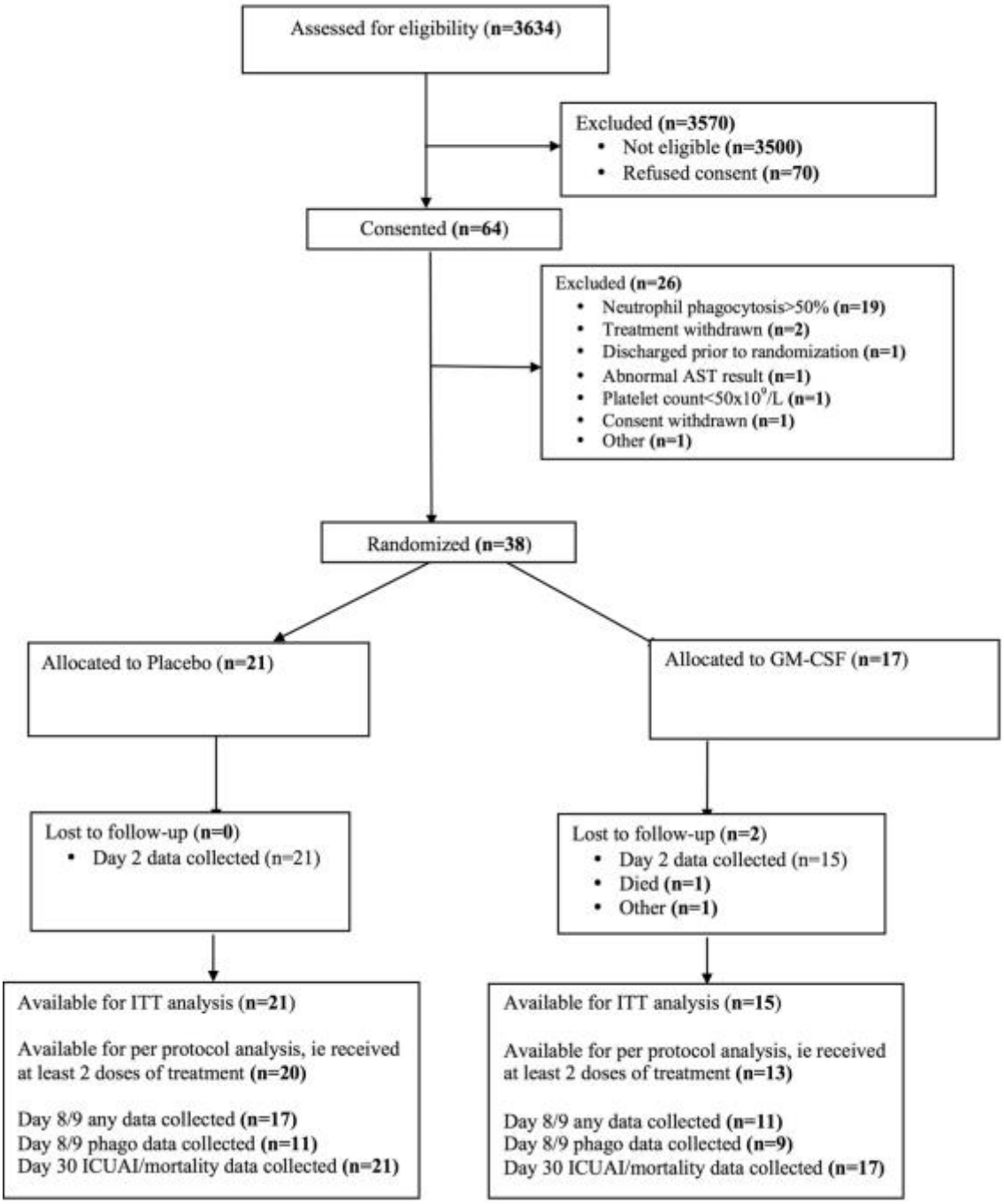

Figure 5.1: CONSORT Diagram of randomised controlled trial. For the two patients excluded from the trial following consent, on the basis of abnormal blood results, the results were found to have become abnormal on routine clinical testing after consent had been taken but prior to randomisation. ITT, intention to treat; ICUAI, ICU acquired infection.

5.9 Baseline demographic data

Overall baseline demographic data were similar between the 2 groups (Table 17). There were more male patients and more patients with a surgical diagnosis in the placebo group compared to the GM-CSF group.

	Placebo	GM-CSF
N	21	17
Median age, years (range)	68 (31-80)	69 (28-89)
Gender (male : female)	15:6	10:7
Median weight, kg (range)	82 (45-144)	77 (49-103)
% with surgical reason for admission to ICU	28.6	17.6
Median APACHE II score (IQR)	21 (18-23)	19.5 (16-27.5)
Sepsis at admission (n)	9	8
Median SOFA Score on admission (IQR)	8 (6-10)	9 (4-11)
Median lowest PaO_2:FiO_2 , kPa (IQR)	25 (17-38)	20 (16-26)
Median lowest MAP, mmHg (IQR)	63 (61-67)	61 (59-65)
Smoking status (current: ex: never: unknown) (%)	19:33:19:29	35:35:24:6
Neutrophil phagocytosis %	40.1 (8.2)	45.1 (4.6)
Median white cell count [$x10^9$/L] (IQR)	11.9 (5.9-12.7)	14.5 (9.6-22.1))
Median neutrophil count [$x10^9$/L] (IQR)	9.5 (4.0-10.8)	13.1 (8.3-20.6)
Median platelet count [$x10^9$/L] (IQR)	164 (101-236)	208 (94-257)
Median creatinine [µmol/L] (IQR)	132 (72-155)	83 (54-159)
Median ALT [U/L] (IQR)	27 (16-36)	27 (17-50)
Median AST [U\L] (IQR)	34 (26-108)	71 (42-92)

Table 21. Baseline demographic data for participants in randomised controlled trial.
ALT, alanine aminotransferase; APACHE, acute physiology and chronic health evaluation; AST, aspartate aminotransferase; FiO_2 inspired concentration of oxygen; ICU, intensive care unit; IQR, inter-quartile range; kg, kilogram; L, litre; MAP, mean arterial pressure; µmol, micromole; PaO_2 partial pressure of oxygen; SOFA, sequential organ failure assessment score, s.d., standard deviation; U, unit.

There was a greater number of patients admitted following emergency surgery within the placebo group compared to the GM-CSF group (Table 18). Otherwise the source of admission was similar between the two groups.

Allocation Arm	Elective medical	Elective surgical	Emergency medical	Emergency surgical
Placebo	1	2	14	4
GM-CSF	0	2	14	1
Total	1	4	28	5

Table 22: Case mix of patients in randomised controlled trial

There were similar rates of co-morbid disease in both groups of patients. Increased rates of known heart disease (angina, congestive heart failure and myocardial infarction) were observed in the placebo group.

	Placebo (Y:N)	GM-CSF (Y/N)
Arthritis	2 (11%) : 17 (89%)	4 (25%) : 12 (75%)
Osteoporosis	0 (0%) : 19 (100%)	0 (0%) : 15 (100%)
Asthma	1 (5%) : 18 (95%)	2 (13%) : 13 (87%)
COPD/ARDS/Emphysema	5 (25%) : 15 (75%)	4 (24%) : 13 (76%)
Angina	2 (21%) :15 (79%)	1 (6%) : 15 (94%)
Congestive heart failure	4 (20%) : 16 (80%)	0 (0%) : 15 (100%)
Myocardial infarction	5 (26%) : 14 (74%)	1 (7%) : 14 (93%)
Neurological disease	0 (0%) : 19 (100%)	0 (0%) : 15 (100%)
Cerebrovascular disease	3 (15%) : 17 (85%)	1 (7%) : 13 (93%)
Peripheral vascular disease	2 (11%) : 17 (89%)	1 (7%) : 13 (93%)
Diabetes type 1 and 2	5 (26%) : 14 (74%)	3 (20%) : 12 (80%)
Upper GI disease	2 (11%) : 17 (89%)	4 (25%) : 12 (75%)
Depression	3 (20%) : 16 (84%)	3 (20%) : 12 (80%)
Anxiety/panic disorders	0 (0%) : 19 (100%)	2 (13%) : 13 (87%)
Visual impairment	2 (11%) : 17 (89%)	0 (0%) : 15 (100%)
Hearing impairment	1 (5%) : 18 (95%)	0 (0%) : 15 (100%)
Obesity and/ or BMI>30	3 (17%) : 15 (83%)	2 (13%) : 13 (87%)
Degenerative disc disease	0 (0%) : 19 (100%)	0 (0%) : 15 (100%)

Table 23: Co-morbid disease data

5.10 Study drug administration

All patients in the RCT were intended to receive a daily injection of subcutaneous GM-CSF at a dose of 3 microgram/kg/day or an equivalent volume of normal saline until one of the study drug termination criteria was met (Chapter 2, section 2.11.5). As outlined in the trial methods (Chapter 2, section 2.11.3) the syringe and contents of the subcutaneous injections of GM-CSF and normal saline were identical in appearance and volume at the point of administration.

The study drug had to be terminated in five patients prior to assessment of the primary outcome on day 2. In the cohort of patients randomised to receive placebo one patient triggered the study drug termination criteria on the morning of day 1 (development of thrombocytopenia). As a result, this patient only received one dose of the study drug during the course of the trial. In the cohort of patients randomised to receive GM-CSF one patient triggered study drug termination criteria prior to receiving any study drug (the patient developed marked thrombocytosis, platelet count 1099 $x10^9$/L) and was discharged from ICU on day 1, with no further blood samples collected. A further three patients in the GM-CSF group triggered study drug termination criteria after only one dose (one patient died, one developed a significant transaminitis and the third developed thrombocytopenia). As a result, only 13 patients in the GM-CSF group received each daily dose of study drug up to and including the point of assessment of the primary outcome (i.e. at least 2 doses of GM-CSF) compared to 20 patients receiving placebo.

Median weight in the GM-CSF group was 77 kg (range 49 – 103) and in the placebo group 82kg (range 45 – 144). The dose or volume of GM-CSF or placebo to be administered was prescribed according to weight ranges to the nearest 5kg. GM-CSF was dosed on actual body weight up to a maximum dose of 450 micrograms /1.8ml by volume (see table 20). Normal saline was prescribed by equivalent volume (see table 21). Corresponding doses or equivalent volumes of study drug ranged from 135 micrograms (0.54ml) to 435 micrograms (1.75ml). In line with the size and graduation of the syringes used to administer the injections, doses above 1ml were rounded to the nearest 0.05mls due to the accuracy with which the dose could be drawn up.

Patient Weight (kg)	Dose of GM-CSF to be administered per day (microgram) (3microgram/kg)	Volume of GM-CSF to be administered subcutaneously per day (ml)	Number of vials required per day
40 (37.5 – 42.4 kg)	120	0.48	1
45 (42.5 – 47.4 kg)	135	0.54	1
50 (47.5 – 52.4 kg)	150	0.60	1
55 (52.5 – 57.4 kg)	165	0.66	1
60 (57.5 - 62.4 kg)	180	0.72	1
65 (62.5 – 67.4 kg)	195	0.78	1
70 (67.5 – 72.4 kg)	210	0.84	1
75 (72.5 – 77.4 kg)	225	0.90	1
80 (77.5 – 82.4 kg)	240	0.96	1
85 (82.5 – 87.4 kg)	255	1.0*	1
90 (87.5 – 92.4 kg)	270	1.1	2
95 (92.5 – 97.4 kg)	285	1.15	2
100 (97.5 – 102.4 kg)	300	1.2	2
105 (102.5 – 107.4 kg)	315	1.25	2
110 (107.5 – 112.4 kg)	330	1.3	2
115 (112.5 – 117.4 kg)	345	1.4	2
120 (117.5 – 122.4 kg)	360	1.45	2
125 (122.5 – 127.4 kg)	375	1.5	2
130 (127.5 – 132.4 kg)	390	1.55	2
135 (132.5 – 137.4 kg)	405	1.6	2
140 (137.5 – 142.4 kg)	420	1.7	2
145 (142.5 – 147.4 kg)	435	1.75	2
150 (147.5 – 150 kg+)	450	1.8	2

Table 24: Dosing schedule for patients allocated to GM-CSF arm

kg, kilogram, ml, millilitres. *In line with the size and graduation of the syringes used to administer the injections, doses above 1ml were rounded to the nearest 0.05mls due to the accuracy with which the dose could be drawn up.

Patient Weight (kg)	Volume of 0.9 % sodium chloride to be administered subcutaneously per day (ml)	Number of ampoules required per day
40 (37.5 – 42.4 kg)	0.48	1
45 (42.5 – 47.4 kg)	0.54	1
50 (47.5 – 52.4 kg)	0.60	1
55 (52.5 – 57.4 kg)	0.66	1
60 (57.5 - 62.4 kg)	0.72	1
65 (62.5 – 67.4 kg)	0.78	1
70 (67.5 – 72.4 kg)	0.84	1
75 (72.5 – 77.4 kg)	0.90	1
80 (77.5 – 82.4 kg)	0.96	1
85 (82.5 – 87.4 kg)	1.0*	1
90 (87.5 – 92.4 kg)	1.1	1
95 (92.5 – 97.4 kg)	1.15	1
100 (97.5 – 102.4 kg)	1.2	1
105 (102.5 – 107.4 kg)	1.25	1
110 (107.5 – 112.4 kg)	1.3	1
115 (112.5 – 117.4 kg)	1.4	1
120 (117.5 - 122.4 kg)	1.45	1
125 (122.5 – 127.4 kg)	1.5	1
130 (127.5 – 132.4 kg)	1.55	1
135 (132.5 – 137.4 kg)	1.6	1
140 (137.5 – 142.4 kg)	1.7	1
145 (142.5 – 147.4 kg)	1.75	1
150 (147.5 – 150kg +)	1.8	1

Table 25: Dosing schedule for patients allocated to placebo arm

kg, kilogram, ml, millilitres. *In line with the size and graduation of the syringes used to administer the injections, doses above 1ml were rounded to the nearest 0.05mls due to the accuracy with which the dose could be drawn up.

5.11 Blood sampling

As described in chapter 2 (section 2.12), blood samples were collected from patients on alternate mornings for assessment of neutrophil function, monocyte and T lymphocyte analysis, and cytokine profiling. Samples for assessment of these biological outcomes were collected from all patients on day 0 and day 2 and then on day 4 or 5, day 6 or 7 and day 8 or 9 according to the availability of the researchers (see table 26). Blood for assessment of these parameters was not collected on weekend days when no researcher was available. As far as possible following discharge of patients from the intensive care unit blood samples continued to be collected at the required time points up until day 8/9 or the point of discharge from hospital, whichever came first. No blood samples were collected from patients following discharge from hospital.

Blood was collected for the assessment of safety parameters on a daily basis, including weekends, as part of usual clinical care.

Missing samples occurred in all data subsets (a) following patient death, (b) in some circumstances following discharge from the ICU, and (c) on weekend days when no researcher was available. In addition, there were some instances of failed assays. Table 22 summarises the completeness of neutrophil phagocytosis data for all patients from day 0 to day 9. Where there are omissions the reason for the omission is described. In circumstances where researcher availability was limited priority was given to completion of the neutrophil phagocytic capacity assay. As a consequence, some of the n values for other neutrophil functional assessments and flow cytometry analyses may be lower than the corresponding n value for the phagocytosis results for any given day.

Patient study ID	DO	D2	D4/5	D6/7	D8/9
F04	X	X	X	X	X
F05	X	X	X	X	X
F06	X	X	X	X	W/E-RNA
F08	X	X	Died D4		
Q07	X	X	X	X	X
Q12	X	Discharged	ICU D1	PNS	
Q15	X	X	X	X	PNS
Q20	X	X	X	Died D5	
Q22	X	X	X	X	X
Q23	X	X	X	X	W/E-RNA
Q24	X	X	W/E-RNA	X	X
Q25	X	X	W/E-RNA	X	X
Q26	X	X	Died D3		
Q27	X	X	W/E-RNA	X	Dis hospital D8
R11	X	X	W/E-RNA	X	X
R13	X	X	X	X	X
R14	X	X	X	X	X
R15	X	X	X	Discharged	Hospital D6
R16	X	X	X	X	X
R18	X	X	X	Died D4	
R21	X	Died D1			
R22	X	X	X	X	Died D7
R24	X	X	X	X	W/E-RNA
R26	X	X	X	Died D5	
R27	X	X	X	X	W/E-RNA
R29	X	X	X	X	X
R30	X	X	X	X	X
R31	X	X	Discharged	hospital	D1
R33	X	X	X	PNS	W/E-RNA
S01	X	X	X	PNS	X
S02	X	X	X	X	X
S03	X	X	X	X	X
S04	X	X	X	X	X
S06	X	X	X	X	X
S07	X	X	X	X	X
S11	X	X	X	Discharged	ICU D7 PNS
S12	X	X	X	X	X
S13	X	X	W/E-RNA	X	X

Table 26: Study day blood sample analysis and missing data

W/E-RNA, weekend-researcher not available; X, sample collected; dis, discharged; PNS, patient not sampled

5.12 Clinical data collection

Clinical data collection included the following parameters; SOFA score, length of ICU and hospital stay, mortality, duration of mechanical ventilation, PaO_2:FiO_2 ratio, body temperature and clinical blood results. These data were recorded on source data (ICU observation charts, hospital clinical notes, ICU blood result charts and electronic patient records) by members of the usual clinical team as part of routine clinical care. All members of the routine clinical team were fully blinded to patient allocation at all times. Members of the research nursing team were involved in the transcription of these data from source data to the electronic case report form.

5.13 Blinding

The study was designed to be a double blind randomised controlled trial with the participants, the research team and the usual clinical team all remaining blinded to the treatment allocation at all times. As discussed in Chapter 2 (section 2.10) complexities in relation to the drug stability following reconstitution and the operating procedures of the clinical trial pharmacies, alongside the absence of a custom made fully matched placebo, resulted in significant logistical challenges in implementing the blinding. A pool of unblinded research nursing staff were recruited and trained to reconstitute and administer the study drug. Reconstitution and administration of the study drug was the only unblinded task undertaken in the delivery of the trial. All other trial procedures were fully blinded at all times. Blinded and unblinded tasks could not be undertaken by the same member of the research nursing team for a given patient to ensure that there was no potential for bias within the results.

Laboratory staff carrying out all laboratory assessments including neutrophil, monocyte, T lymphocyte and cytokine analysis for primary and secondary outcomes were fully blinded to sample treatment allocation at all times.

Following completion of the study, during the processes of internal and external audits it became apparent that at two sites research nursing staff who had been involved in the unblinded reconstitution and administration of the study drug, had taken part in the blinded task of transfer of clinical data from source data to the electronic case report form for the same patient. As the clinical data were all recorded independently at source, by the clinical nursing team or hospital laboratory

as part of usual clinical care, the potential for bias (through the participation of an unblinded member of the research nursing team in this task) is considered to be negligible but could not be fully excluded. As a consequence, the Research Sponsor (Newcastle upon Tyne Hospitals NHS Foundation Trust) and the MHRA recommended that the study should be reported as a single blinded trial.

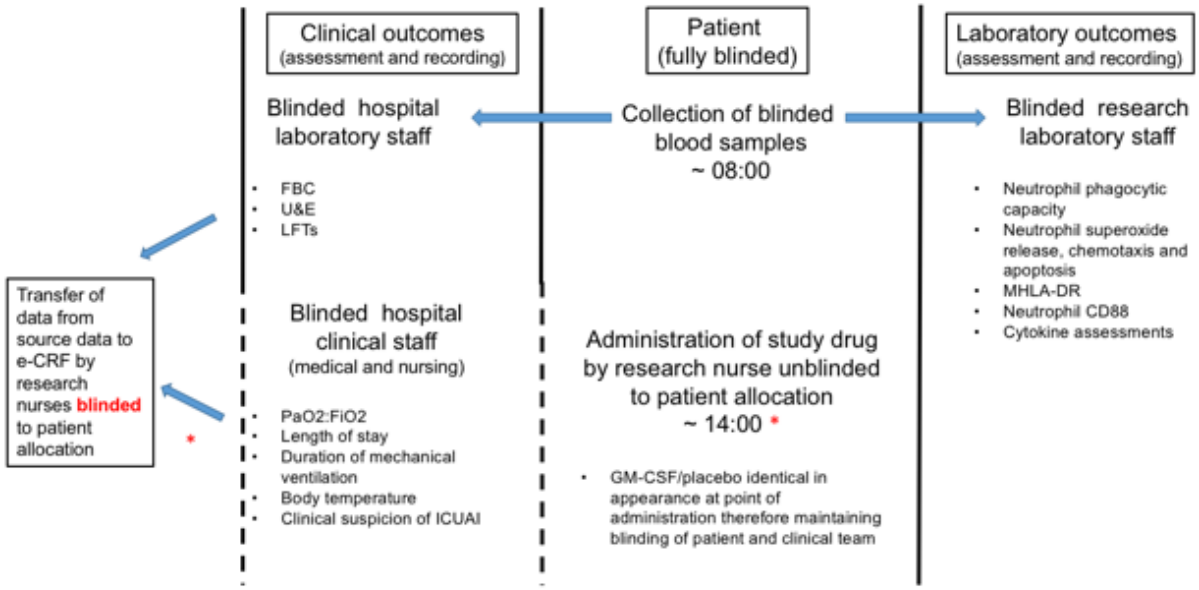

Figure 5.2: Schematic diagram showing mechanisms of blinding and potential breach of blinding. The patient, research laboratory staff, hospital laboratory staff and hospital clinical staff remained fully blinded at all times. The study drug was administered by research nurses unblinded to the allocation for that particular patient. During audit processes it became apparent that research nursing staff, unblinded to the treatment allocation of an individual patient, had participated in the blinded task of transfer of source data to the e-CRF for the same patient. This potential breach had occurred in patients at two sites. * Source of potential breach of blinding - research nursing staff were found to have undertaken the blinded task of transfer of clinical data on patients in whom they had administered study drug even though it had been mandated that they should not. Solid black line signifies complete separation of participant / staff at all times. Dotted black line indicates staff working within same environment at certain times.

5.14 Results: Neutrophil phagocytic capacity

As previously described in chapter 2 (section 2.16) neutrophils were extracted from whole blood by dextran sedimentation and percoll gradient separation technique. Following isolation neutrophils were pre-incubated with autologous serum and then incubated with zymosan particles for 30 minutes at 37°C in a 5% CO_2 incubator. Neutrophil phagocytic capacity was assessed by light microscopy by counting the percentage of neutrophils ingesting 2 or more zymosan particles.

5.14.1 Neutrophil phagocytic capacity at baseline

There was an unexpected difference seen in neutrophil phagocytic capacity between the two groups at baseline with mean phagocytic capacity being 40.1 (s.d.+/- 8.2, n=21) in the group subsequently randomised to placebo group versus mean of 45.1 (s.d. +/- 4.6, n=17) in the group randomised to receive GM-CSF. This difference was statistically significant (p=0.03). Where analyses have been found to be statistically significant adjustment for baseline will be carried out.

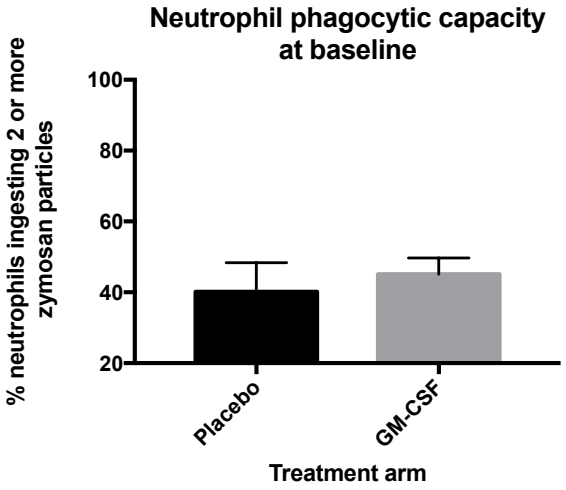

Figure 5.3: Mean neutrophil phagocytic capacity at baseline. A statistically significant difference was seen at baseline between the two groups when analysed by 2-sample t-test. Mean placebo 40.1% (s.d.+/- 8.2, n= 21), mean GM-CSF 45.1% (s.d.+/- 4.6, n= 17), p=0.03.

5.14.2 Neutrophil phagocytic capacity on day 2

A rise in neutrophil phagocytic capacity was seen between day 0 and day 2 (the day for assessment of the primary outcome) in both groups. Mean neutrophil phagocytic capacity in the placebo group was 49.8% (s.d.+/- 13.4) versus mean neutrophil phagocytic capacity in the GM-CSF group of 57.2% (s.d.+/- 13.2), p=0.11. The mean rise in GM-CSF treated patients was 11.9% (s.d. +/- 12.3%, n=15) versus 9.6% (s.d. +/- 10.8%, n=21) in the placebo group. There was no significant difference seen in neutrophil phagocytic capacity at day 2, however.

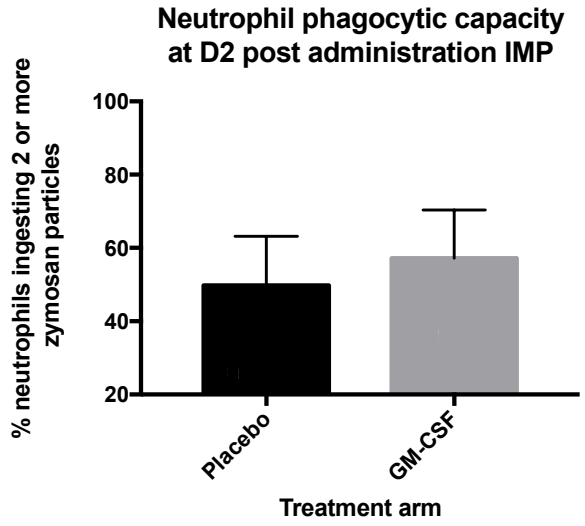

Figure 5.4: Mean neutrophil phagocytic capacity at day 2. No significant difference was seen between the two groups on day 2 when analysed by 2-sample t-test. Mean placebo 49.8% (s.d.+/- 13.4, n= 21), mean GM-CSF 57.2% (s.d.+/- 13.2, n= 15), p=0.11.

5.14.3 Neutrophil phagocytic capacity on day 4/5

There was a statistically significant difference seen in neutrophil phagocytic capacity between the 2 groups at day 4/5 - mean in the GM-CSF group 62.3 %(+/- 15.7, n=12) versus mean placebo group 50.3% (+/- 14.3, n=16), p=0.046. This significance was lost however, when adjusted for site and baseline neutrophil phagocytic capacity (p=0.15). The mean rise in neutrophil phagocytic capacity was 17.2% (+/- 13.6) in the GM-CSF group versus 10.8% (+/- 14.1) in the placebo group.

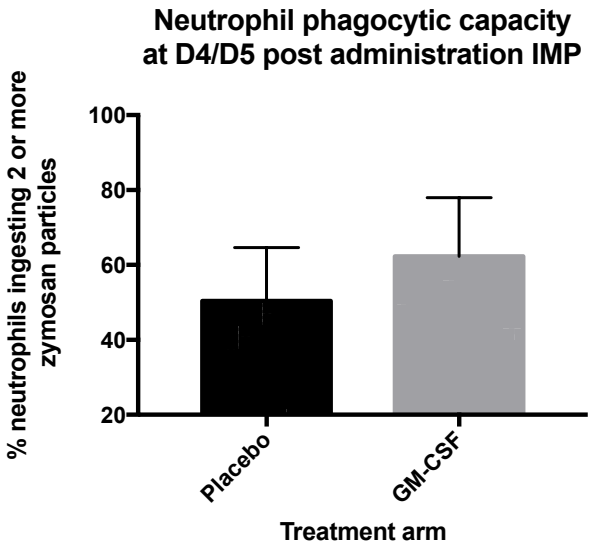

Figure 5.5: Mean neutrophil phagocytic capacity at day 4/5. A statistically significant difference was seen between the two groups on day 4/5 when analysed by 2-sample t-test. Mean placebo 50.3% (s.d.+/- 14.3, n= 16), mean GM-CSF 62.3% (s.d.+/- 15.7, n= 12), p=0.046.

5.14.4 Neutrophil phagocytic capacity on day 6/7

Mean neutrophil phagocytic capacity was once again seen to be greater in the GM-CSF treated group compared to the placebo group on day 6/7. However, this fell just outside statistical significance, p=0.05. Mean neutrophil phagocytic capacity was 64% (+/- 11.4, n=10) in the GM-CSF group with mean capacity of 52.7% (+/- 15.0, n=16) in the placebo group.

5.14.4 Neutrophil phagocytic capacity on day 8/9

Finally, on day 8/9 there was no significant difference observed in neutrophil phagocytic capacity between the 2 groups. Mean neutrophil phagocytic capacity was 57.2% (s.d. +/- 16.6, n=11) in the placebo group versus 68.3% (s.d.+/- 9.1, n=9) in the GM-CSF group (p=0.09).

Study Day	Statistical indices	Placebo	GM-CSF
Baseline : Day 0	N Mean S.D. Median Range IQR	21 40.1 8.2 41.3 15.2 – 48.7 36.3 – 47.3	17 45.1 4.6 46.6 35.1 – 49.7 42.4 – 48.7
Day 2	N Mean S.D. Median Range IQR	21 49.8 13.4 48.8 23 – 77.1 41.7 – 59.6	15 57.2 13.2 60.8 22.4 – 76.3 51.9 – 76.3
Day 4/5	N Mean S.D. Median Range IQR	16 50.3 14.3 47.1 33.9 – 86 40.1 – 56.8	12 62.3 15.7 63.2 29.6 – 86.1 49.5 – 74.8
Day 6/7	N Mean S.D. Median Range IQR	16 52.7 15.0 48.9 34.6 – 88.3 40.9 – 62.2	10 64.0 11.4 63.5 51.5 – 86.2 52.9 – 71.5
Day 8/9	N Mean S.D. Median Range IQR	11 57.2 16.6 59.2 32 - 83.2 44.2 – 72.7	9 68.3 9.1 66.7 53.2 84.9

Table 27: Summary statistics for neutrophil phagocytic capacity over study course

5.14.5 Proportion of patients with adequate neutrophil phagocytic capacity

There was a statistically significant difference seen in the proportion of patients with adequate neutrophil phagocytic capacity (i.e. ≥ 50% of neutrophils ingesting 2 or more zymosan particles following a zymosan "meal") in the GM-CSF group compared with the placebo group on day 2 (42.9% versus 80%, p=0.041) and day 6/7 (43.8% versus 100%, p=0.004). On day 4/5 and day 8/9, although there was a greater proportion of patients with adequate neutrophil phagocytic capacity, this did not reach statistical significance (day 4/5 placebo 43.8% versus GM-CSF 75%, p=0.13, day 8/9 placebo 63.6% versus GM-CSF 100%, p=0.09).

5.14.5.1 Proportion of patients with adequate neutrophil phagocytic capacity on day 2

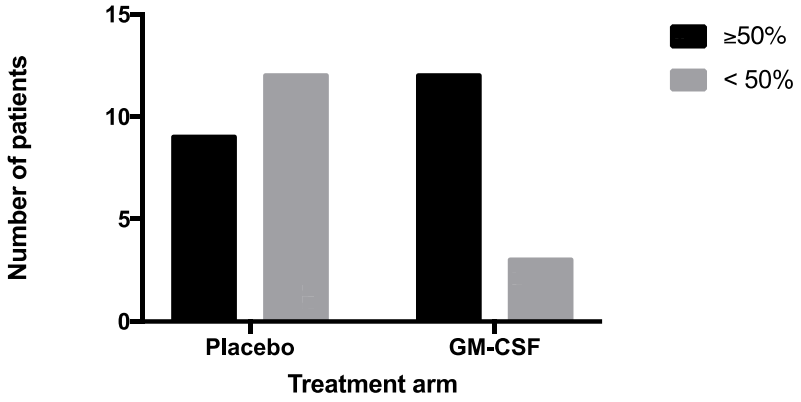

Figure 5.6: Proportion of patients with neutrophil phagocytic capacity ≥50% or <50%on day 2. There was a significant difference was seen between the two groups on day 2 when analysed by Fisher's exact test. % with adequate neutrophil phagocytic capacity (≥ 50%) placebo 42.9% (9 of 21 patients) versus GM-CSF 80% (12 of 15 patients) (p=0.041).

5.14.5.2 Proportion of patients with adequate neutrophil phagocytic capacity on day 4/5

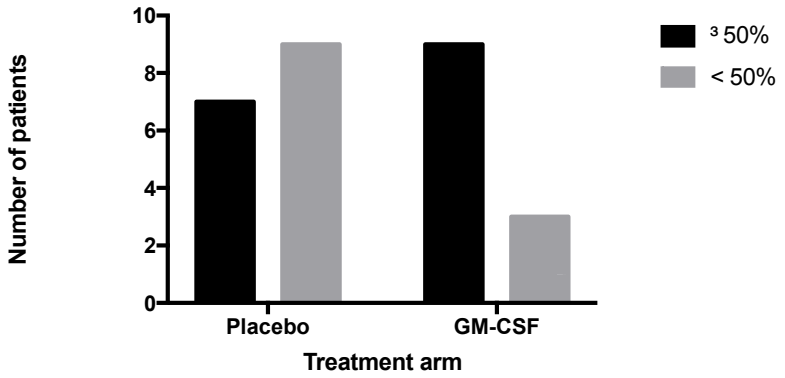

Figure 5.7: Proportion of patients with neutrophil phagocytic capacity ≥50% or <50%on day 4/5. There was no significant difference seen between the two groups on day 4/5 when analysed by Fisher's exact test. % of patients with adequate neutrophil phagocytic capacity (i.e. ≥ 50%) placebo 43.8% (7 of 16 patients) versus GM-CSF 75% (9 of 12 patients) (p=0.14).

5.14.5.3 Proportion of patients with adequate neutrophil phagocytic capacity on day 6/7

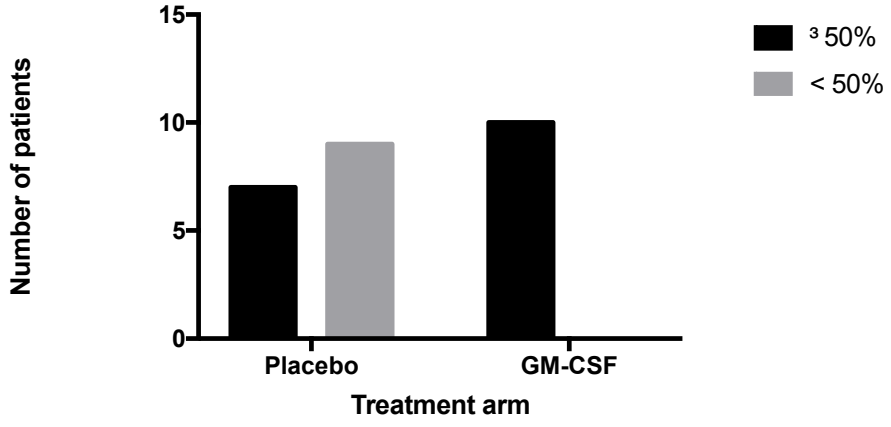

Figure 5.8: Proportion of patients with neutrophil phagocytic capacity ≥50% or <50%on day 6/7. There was a significant difference seen between the two groups on day 6/7 when analysed by Fisher's exact test. % of patients with adequate neutrophil phagocytic capacity (i.e. ≥ 50%) placebo 43.8% (7 of 16 patients) versus GM-CSF 100% (10 of 10 patients) (p=0.004).

5.14.5.4 Proportion of patients with adequate neutrophil phagocytic capacity on day 8/9

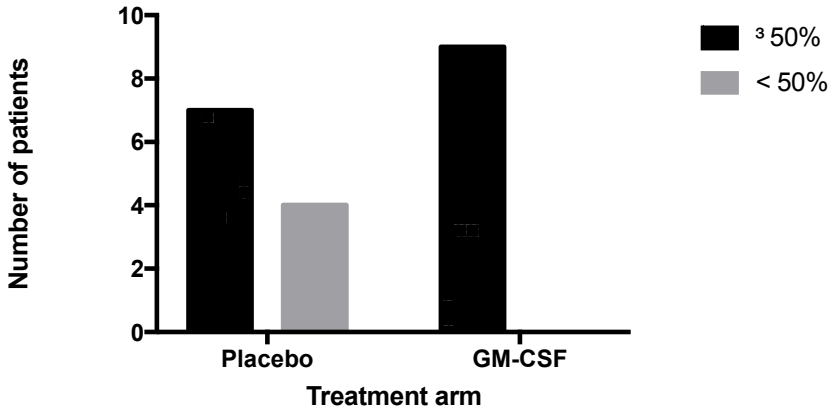

Figure 5.9 Proportion of patients with neutrophil phagocytic capacity ≥50% or <50%on day 8/9. There was no significant difference seen between the two groups on day 8/9 when analysed by Fisher's exact test. Patients with adequate neutrophil phagocytic capacity (≥ 50%) placebo 63.6% (7 of 11 patients) versus GM-CSF 100% (9 of 9 patients) (p=0.09).

5.14.6 Area under the curve for neutrophil phagocytic capacity up to day 8/9

There was a statistically significant difference in the area under the curve between D0 and D8/9 between the two groups, p=0.011. When adjusted for baseline phagocytosis and site, however, this significance was lost, p =0.14. The curves were seen to diverge in particular up to day 4/5.

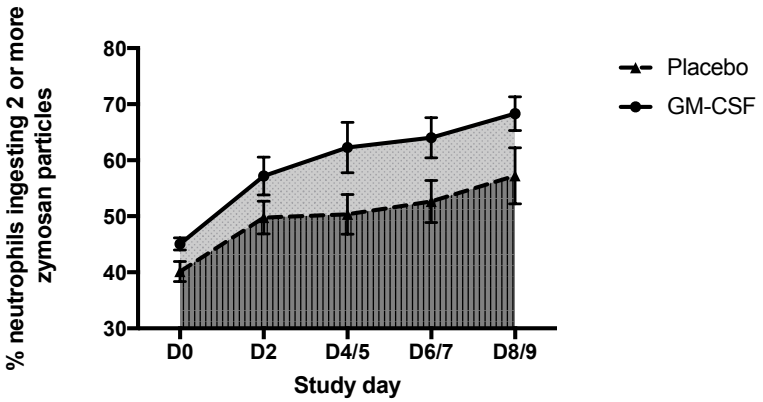

Figure 5.10: Area under the curve for neutrophil phagocytic capacity up to day 8/9. A significant difference was seen between the area under the curve for the two groups up to day 8/9. GM-CSF mean 553.9 (+/- 73.5, n=9) versus placebo mean 451.9 (+/- 85.2, n= 11) when analysed by 2 sample t-test. p=0.011. Symbol = mean, whiskers = S.E.M.

5.14.7 Neutrophil superoxide release

As described in chapter 2 (section 2.16.6) following isolation from whole blood neutrophils were re-suspended in HBSS+, primed with PAF and incubated at 37ºC in a shaking water bath. Following stimulation with fMLF, in the presence of superoxide dismutase and cytochrome C, the generation of superoxide anion (O_2^-) was calculated by determining the amount of superoxide dismutase-inihibitable reduction of cytochrome C. Results were expressed as nanomoles of superoxide anions generated per million neutrophils (nmolO_2^-/10^6 neuts)

5.14.7.1 Neutrophil superoxide release at baseline

There was no significant difference in neutrophil superoxide release at baseline between the two groups. Mean superoxide release in the placebo group was 2.15 nmol O_2^-/10^6 neuts (s.e.m. +/- 0.45) versus 1.98 nmol O_2^-/10^6 neuts (s.e.m, +/- 0.39) in the GM-CSF group (p=0.78).

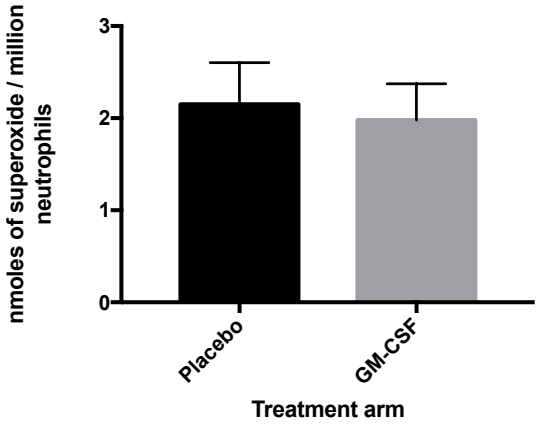

Figure 5.11: Mean neutrophil superoxide release at baseline. There was no significant difference seen between the two groups at baseline when analysed by 2 sample t-test. Placebo mean 2.15 nmol O_2^-/10^6 neuts (s.e.m. +/- 0.45, n= 19), GM-CSF mean 1.98 nmol O_2^-/10^6 neuts (s.e.m +/- 0.39, n=15) (p=0.78).

5.14.7.2 Neutrophil superoxide release on day 2

There was no significant difference in neutrophil superoxide release between the two groups on day 2 following administration of the study drug. Mean superoxide release in the placebo group was 2.05 nmol $O_2^-/10^6$ neuts (s.e.m. +/- 0.34) versus 2.49 nmol $O_2^-/10^6$ neuts (s.e.m, +/- 0.38) in the GM-CSF group (p=0.39).

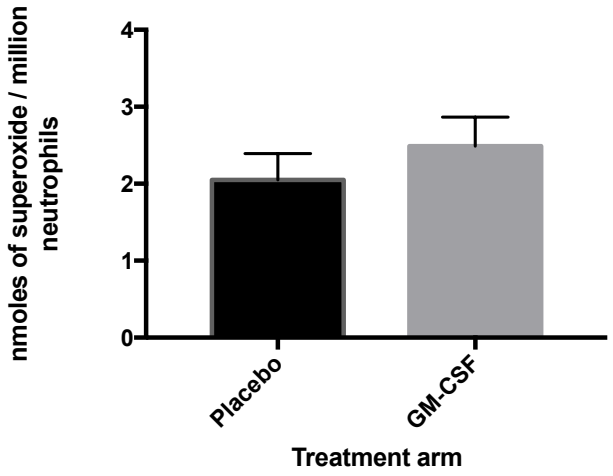

Figure 5.12: Mean neutrophil superoxide release on day 2. There was no significant difference seen between the two groups on day 2 when analysed by 2 sample t-test. Placebo mean 2.05 nmol $O_2^-/10^6$ neuts (s.e.m. +/- 0.34, n=17), GM-CSF mean 2.49 nmol $O_2^-/10^6$ neuts (+/- 0.38, n=15) (p=0.39).

5.14.7.3 Neutrophil superoxide release on day 4/5, day 6/7, day 8/9

There was no significant difference in neutrophil superoxide release between the two groups on any other day following administration of the study drug. Mean superoxide release on day 4/5 in the placebo group was 1.51 nmol $O_2^-/10^6$ neuts (s.e.m. +/- 0.34) versus 2.63 nmol $O_2^-/10^6$ neuts (s.e.m, +/- 0.80) in the GM-CSF group (p=0.20). Equivalent values on day 6/7 were 2.23 nmol $O_2^-/10^6$ neuts (s.e.m. +/- 0.44) in the placebo group versus 2.29 nmol $O_2^-/10^6$ neuts (s.e.m, +/- 0.42) in the GM-CSF group (p=0.92), while on day 8/9 they were 0.68 nmol $O_2^-/10^6$ neuts (s.e.m. +/- 0.14) in the placebo group versus 1.35 nmol $O_2^-/10^6$ neuts (s.e.m, +/- 0.43) in the GM-CSF group (p=0.13)

5.14.8 Neutrophil chemotaxis

As described in chapter 2 (section 2.16.7) following isolation from whole blood neutrophils were re-suspended at $25x10^6$/ml in IMDM containing 1% autologous serum. They were placed in a central well on an agarose-coated slide. Chemoattractant in the form of fMLF was placed in an adjacent well with IMDM being placed in a control well equidistant on the other side. Following incubation at 37ºC/ 5%CO_2 the slides were fixed and stained and the neutrophil migration distance was assessed by computer imaging.

5.14.8.1 Neutrophil chemotaxis at baseline

There was no significant difference in the migratory distance to chemoattractant seen between the two groups at baseline.

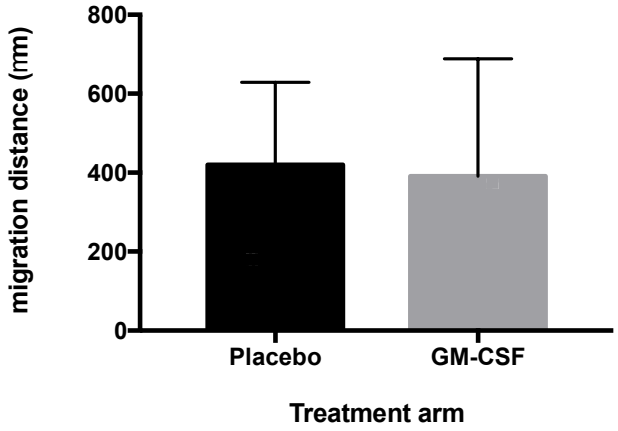

Figure 5.13: Neutrophil chemotaxis at baseline. There was no significant difference seen in the migration distance between the two groups at baseline. Median migratory distance placebo group was 420μm (IQR 0-630, n=15), GM-CSF group was 391μm (IQR 146-689, n=12), p=0.75, Mann Whitney U test.

5.14.8.2 Neutrophil chemotaxis on day 2

There was no significant difference in the migratory distance to chemo attractant seen between the two groups on day 2 following the administration of the study drug.

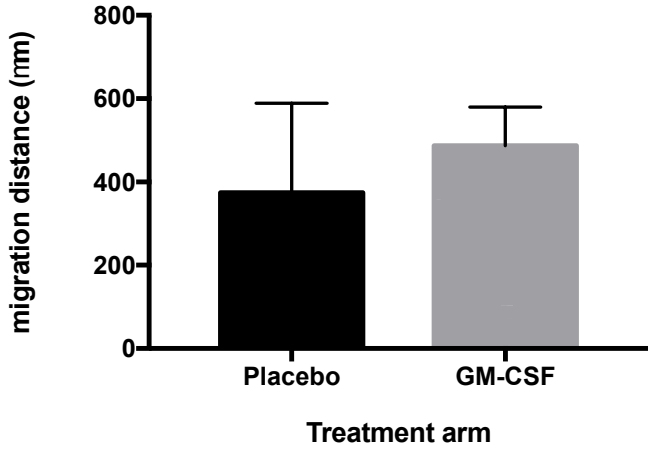

Figure 5.14: Neutrophil chemotaxis on day 2. There was no significant difference seen in the migration distance between the two groups on day 2. Median migration distance placebo group was 374μm (IQR 116-588, n=14), GM-CSF group 487 (IQR 185-580, n=12), p= 0.56, Mann Whitney U test.

5.14.8.3 Neutrophil chemotaxis day 4/5, day 6/7, day 8/9

There was no significant difference in the migratory distance to chemo attractant fMLF seen between the two groups on any other study day. Day 4/5, placebo n=9, GM-CSF n=10, p= 0.66, day 6/7 placebo n=8, GM-CSF n=6, p= 0.56, day 8/9 placebo n=4, GM-CSF n=3 p= 0.23, Mann Whitney U test.

5.14.9 Neutrophil apoptosis

As described in chapter 2 (section 2.16.8) following isolation from whole blood neutrophils were re-suspended in HBSS+ washed once and re-suspended in Annexin V buffer solution. The cells were incubated with Annexin V and subsequently with propidium iodide (PI) in the dark at room temperature. Following incubation cells were washed and re-suspended in Annexin V buffer and analysed by flow cytometry. The proportion of cells showing evidence of early and late apoptosis by Annexin V / PI analysis was calculated.

5.14.9.1 Neutrophil apoptosis at baseline

There was no significant difference seen in the early and late apoptosis rates between the two groups at baseline**.**

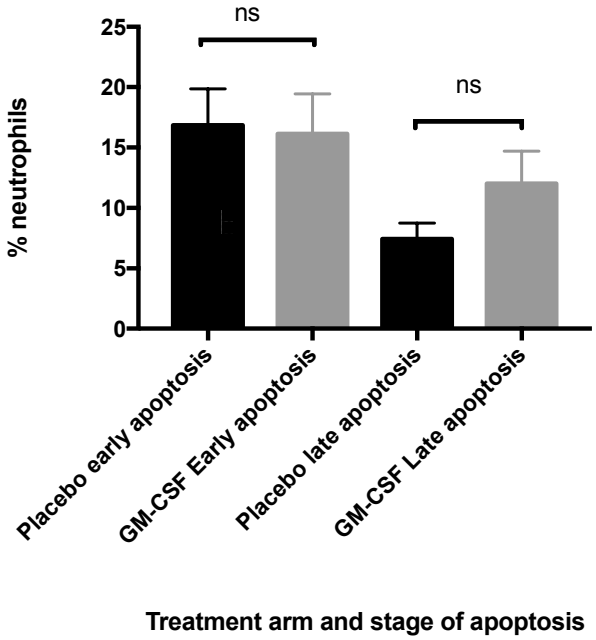

Figure 5.15: Mean neutrophil apoptosis rates at baseline. There was no significant difference seen in the apoptotic rates between the two groups at baseline when analysed by 2-sample t test. Early apoptosis placebo 16.9% (s.e.m +/- 3, n=17) GM-CSF 16.1% (s.e.m. +/- 3.3, n=16) p= 0.88; late apoptosis placebo 7.4% (s.e.m. +/- 1.3, n=17) GM-CSF 12.0% (s.e.m. +/- 2.7, n=16) p = 0.13.

5.14.9.2 Neutrophil apoptosis on day 2

There was no significant difference in the neutrophil apoptotic rate seen between the two groups on day 2 following the administration of the study drug.

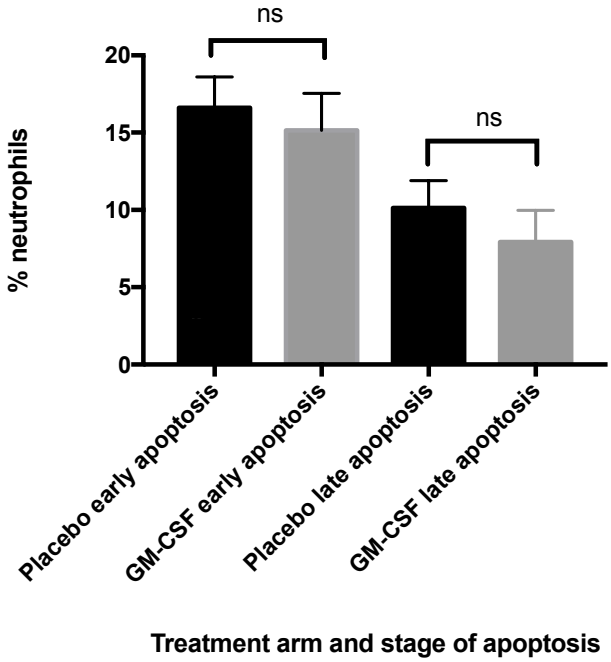

Figure 5.16: Mean neutrophil apoptosis rates on day 2. There was no significant difference seen in the apoptotic rates between the two groups on day 2 when analysed by 2-sample t test. Early apoptosis placebo 16.6% (s.e.m +/- 2, n=17) GM-CSF 15.1% (s.e.m. +/- 2.4, n=15) p= 0.64; late apoptosis placebo 10.1% (s.e.m. +/- 1.8, n=17) GM-CSF 7.9% (s.e.m. +/- 2.1, n=15) p = 0.42.

5.14.9.3 Neutrophil apoptosis day 4/5, day 6/7, day 8/9

There was no significant difference in the neutrophil apoptotic rate seen between the two groups on any other study day, when analysed by 2-sample t test. Day 4/5 - early apoptosis placebo 16.3% (s.e.m. +/- 4.6, n=13) GM-CSF 13.8% (s.e.m. +/- 2.8, n=9) p= 0.67; late apoptosis placebo 6.6% (s.e.m. +/- 1.2, n=13) GM-CSF 5.1% (s.e.m +/- 1.0, n=9) p = 0.37. Day 6/7 early apoptosis placebo 17.6% (s.e.m +/- 3.4, n=15) GM-CSF 15.8% (s.e.m +/- 3.3, n=10) p= 0.72; late apoptosis placebo 7.4% (s.e.m +/- 1.6, n=15) GM-CSF 8.9% (s.e.m. +/- 2.5, n=10) p = 0.61. Day 8/9 early apoptosis placebo 13.8% (s.e.m. +/- 3.8, n=8) GM-CSF 24.1% (s.e.m. +/- 4.5, n=8) p= 0.10; late apoptosis placebo 7.0% (s.e.m. +/- 2.1, n=8) GM-CSF 6.7% (s.e.m. +/- 1.3, n=8) p = 0.92.

5.14.10 Neutrophil CD88 expression

As described in chapter 2 (section 2.16.4) samples of whole blood were collected for flow cytometry analysis including assessment of neutrophil CD88 expression. Whole blood was incubated with phycoerythrin-conjugated antibody or isotype control at 4ºC in the dark. Following incubation red cell lysis was performed with Pharmlyse solution and the cells were washed prior to analysis by flow cytometry. Neutrophils were identified by forward scatter and side scatter characteristics, gated accordingly and CD88 expression was measured in arbitrary units.

5.14.10.1 Baseline neutrophil CD88 expression at baseline

There was no significant difference seen in the neutrophil CD88 expression between the two groups at baseline. Median neutrophil CD88 expression in the placebo group was 559 (IQR 463-914) arbitrary units compared to median of 714 (IQR 541-868) in the group allocated to GM-CSF (p=0.71).

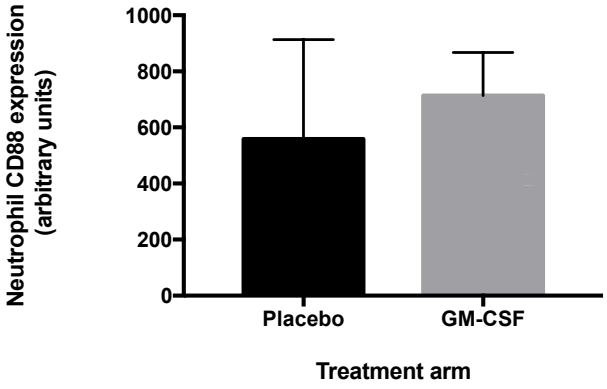

Figure 5.17: Neutrophil CD88 expression at baseline. There was no significant difference seen in the neutrophil CD88 expression between the two groups at baseline when analysed by Mann Whitney U test. Placebo median 559 (IQR 463-914, n=19) versus GM-CSF median 714 (IQR 541-868, n=15) arbitrary units, p= 0.71.

5.14.10.2 Neutrophil CD88 expression on day 2

There was no significant difference seen in neutrophil CD88 expression between the two groups on day 2 following the administration of the study drug. Median neutrophil CD88 expression in the placebo group was 953 (IQR 544-1334) arbitrary units compared to median of 885 (IQR 483-1013) in the group allocated to GM-CSF (p=0.52).

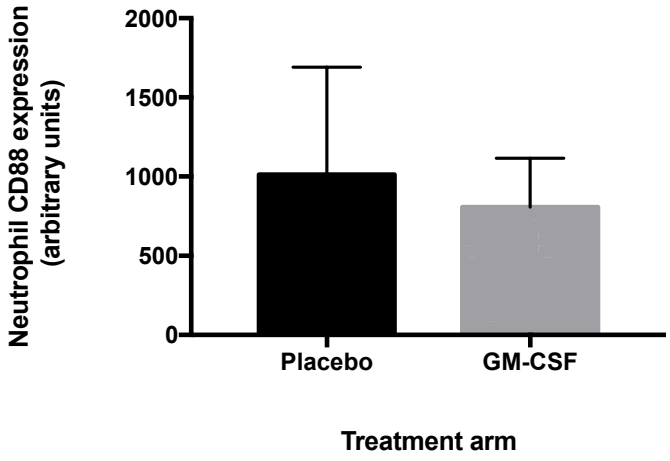

Figure 5.18: Neutrophil CD88 expression on day 2. There was no significant difference seen in the neutrophil CD88 expression between the two groups on day 2 when analysed by Mann Whitney U test. Placebo median 953 (IQR 544-1334, n=18) versus GM-CSF median 885 (IQR 483-1013, n=12) arbitrary units, p= 0.52.

5.14.10.3 Neutrophil CD88 expression day 4/5, day 6/7, day 8/9

There was no significant difference in the neutrophil CD88 expression seen between the two groups on any other study day.

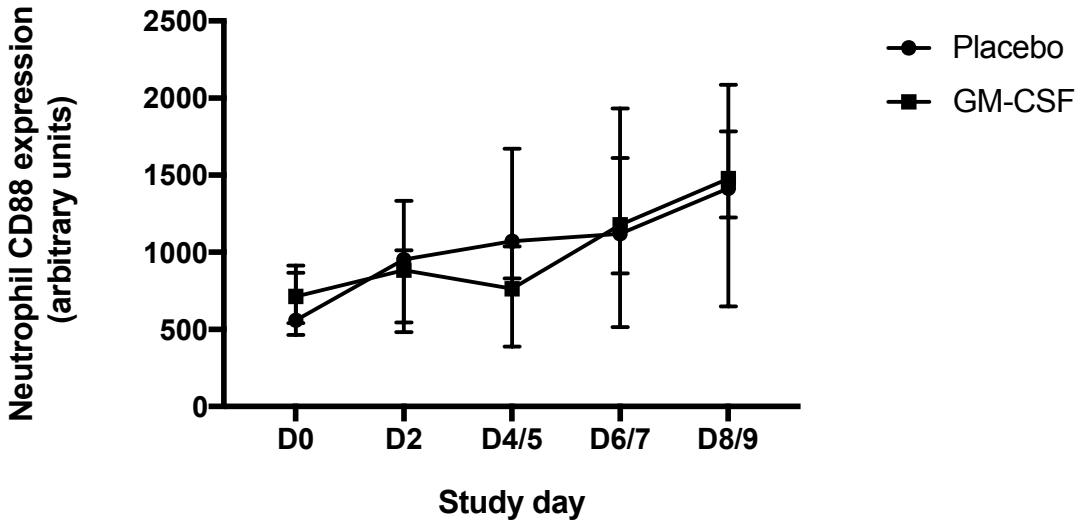

Figure 5.19: Neutrophil CD88 expression over study period. There was no significant difference seen in the neutrophil CD88 expression between the two groups on any other study day. Day 4/5 placebo median 1072 (IQR 830-1671, n=14) versus GM-CSF median 764 (IQR 389-1038, n=8) arbitrary units, p= 0.07. Day 6/7 placebo median 1119 (IQR 864-1932, n=15) versus GM-CSF mean 1178 (IQR 516-1612, n=9) arbitrary units, p= 0.41. Day 8/9 placebo median 1415 (IQR 1227-1785, n=11) versus GM-CSF median 1478 (IQR 648-2086, n=8) arbitrary units, p= 0.97. Mann Whitney U test.

5.14.10.5 Correlation between baseline neutrophil CD88 and neutrophil phagocytic capacity

Contrary to previous findings there was no significant correlation observed between neutrophil CD88 expression and neutrophil phagocytic capacity at baseline.

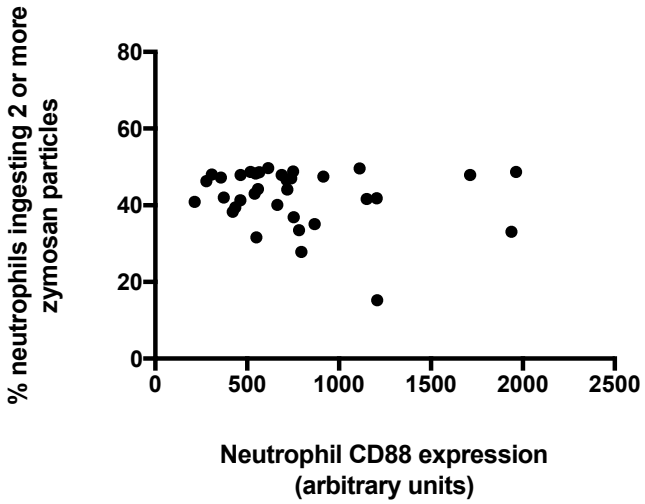

Figure 5.20: Correlation between neutrophil CD88 expression and neutrophil phagocytic capacity at baseline. Spearman's r = - 0.09 (p= 0.622, n=34, pooled data for placebo and GM-CSF groups).

There was a weak correlation seen between neutrophil phagocytic capacity and neutrophil CD88 expression over the course of the study.

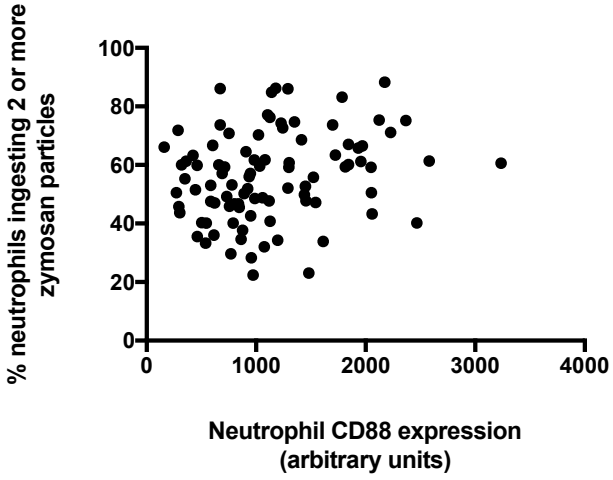

Figure 5.21: Correlation between neutrophil CD88 expression and neutrophil phagocytic capacity over the course of the study. Spearman's r = 0.26 (p= 0.012, n=92, pooled data for placebo and GM-CSF groups).

5.14.11 Monocyte HLA-DR expression

As described in chapter 2 (section 12.15.5) samples of whole blood were collected for flow cytometry analysis including assessment of monocyte HLA-DR expression. Whole blood was incubated with QuantiBRITE^{TM} PE-conjugated antibody at room temperature in the dark. Following incubation red cell lysis was performed with Pharmlyse solution and the cells were washed prior to analysis by flow cytometry. Monocytes were initially identified by forward scatter and side scatter characteristics and then by CD14/ CD64 positivity. The number of antibodies bound per cell (Ab/cell) was calculated.

5.14.11.1 Baseline monocyte HLA-DR expression

There was no significant difference seen in the mean monocyte HLA-DR expression between the two groups at baseline. Mean monocyte HLA-DR expression in the placebo group was 6382 (s.d. +/- 5149) arbitrary units compared to mean of 6179 (s.d. +/- 4146) in the group allocated to GM-CSF (p=0.90).

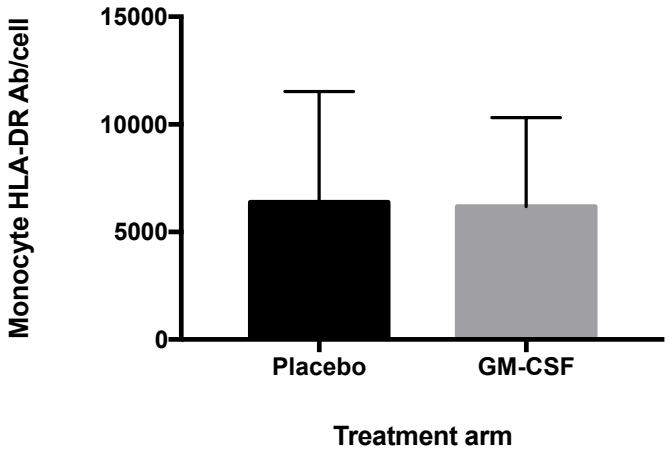

Figure 2.22: Mean monocyte HLA-DR expression at baseline. There was no significant difference seen in monocyte HLA-DR expression between the two groups at baseline when analysed by 2-sample t test. Placebo mean 6382 (s.d.+/- 5149, n=18) versus GM-CSF mean 6179 (s.d. +/- 4146, n=16) arbitrary units, p= 0.90.

5.14.11.2 Monocyte HLA-DR expression day 2

There was a statistically significant difference seen in monocyte HLA-DR expression between the two groups on day 2 following the administration of the study drug. Mean HLA-DR expression in the placebo group was 6097Ab/cell (s.d +/- 4501, n=18) compared with mean HLA-DR expression 54999 Ab/cell (s.d. +/- 31239, n=13) in the GM-CSF group ($p<0.0001$). The mean rise in mHLA-DR expression in the placebo group was -285.3 Ab/cell (s.d. +/- 2529) compared with a mean rise of 48949 Ab/cell (s.d. +/- 29381) in the GM-CSF group.

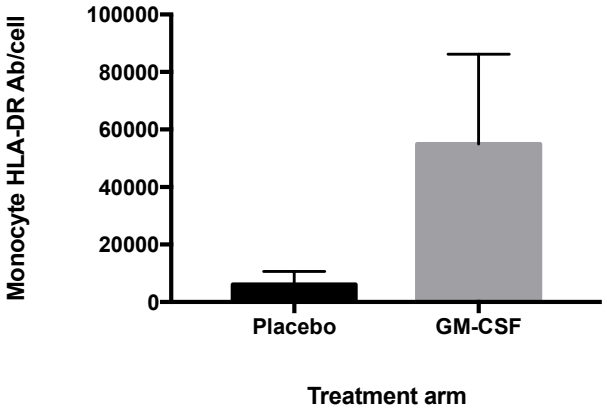

Figure 5.23: Mean monocyte HLA-DR expression on day 2. There was a statistically significant difference seen in absolute monocyte HLA-DR expression seen between the two groups on day 2 when analysed by 2-sample t test. Placebo mean 6097 (s.d.+/- 4501, n=18) versus GM-CSF mean 54999 (s.d. +/- 31239, n=13), $p< 0.0001$.

5.14.11.3 Monocyte HLA-DR expression over study course

A statistically significant difference in monocyte HLA-DR expression continued to be seen between the two groups at day 4/5 - mean mHLA-DR in the placebo group 5807 Ab/cell (s.d. +/- 5303, n=14) versus mean mHLA-DR 30706 Ab/cell (+/- s.d. 34484, n=8) in the GM-CSF group, $p=0.01$. Following the completion of study drug administration on day 3 the levels of mHLA-DR expression were seen to fall in the GM-CSF group reaching the pre-treatment baseline levels by day 6/7.

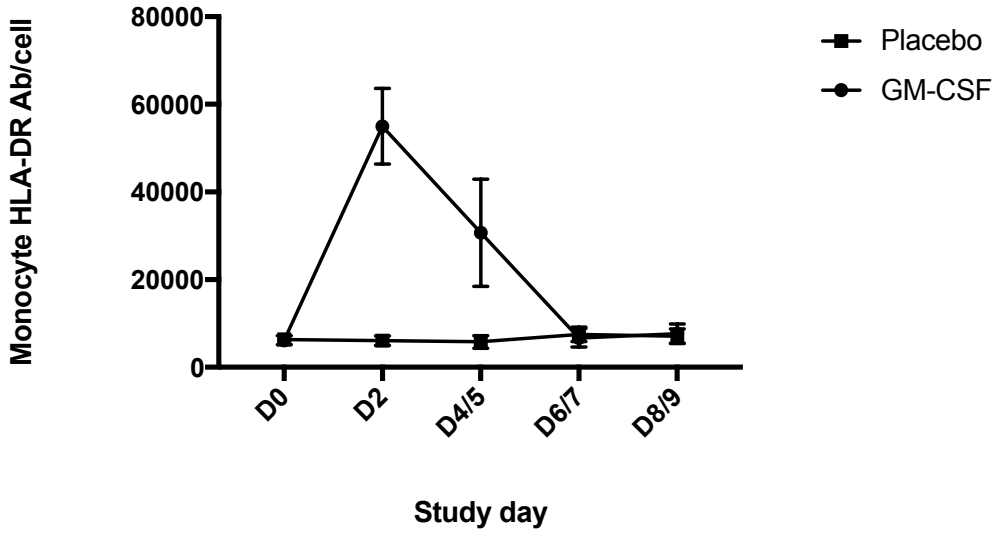

Figure 5.24: Mean monocyte HLA-DR expression over study course. A statistically significant difference continued to be seen in absolute monocyte HLA-DR expression on day 4/5 (placebo 5807 Ab/cell (s.d.+/- 5303, n=14) versus GM-CSF 30706 Ab/cell (s.d.+/- 34484, n=8), p= 0.01). Following this mHLA-DR expression in the GM-CSF group was seen to fall to baseline levels. Day 6/7 p= 0.77 (placebo n= 15, GM-CSF = 9), day 8/9 p= 0.79 (placebo n=12, GM-CSF n=8).

5.14.12 T cell analysis

As described in chapter 2 (section 2.16.9) T cell analysis was performed by flow cytometry to determine regulatory T cell (Treg) proportion as a percentage of $CD4^+$ cells. Whole blood was incubated with the antibody panels described (section 2.16.9) at 4ºC in the dark. Following incubation red cell lysis was performed with Pharmlyse solution and the cells were washed prior to analysis by flow cytometry. Tregs were identified by $CD4^+$, $CD25^+$, $CD127^{low}$ characteristics. Differentiation of memory and naïve cells was performed by CD45RA/CD45RO analysis.

5.14.12.1 Proportion of regulatory T cells at baseline

There was no significant difference seen in the mean Treg proportion between the two groups at baseline, when analysed by two-sample t-test. Mean Treg proportion in the placebo group was 16.6% (s.d. +/- 4.1, range 7.8-22.9%, n=15) compared to

mean of 17.6% (s.d. +/-5.7,range 10.4-27.9%, n=13) in the group allocated to GM-CSF, p=0.62.

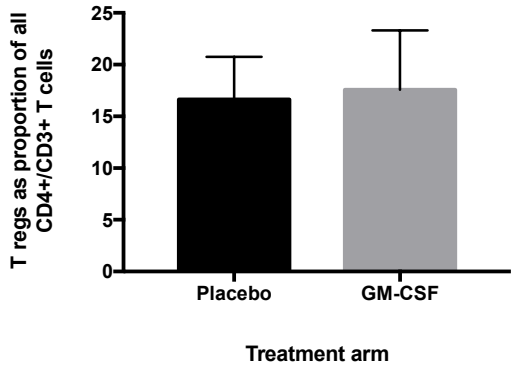

Figure 2.25: Proportion of regulatory T cells (Tregs) at baseline. There was no significant difference seen in the proportion of Tregs between the two groups at baseline when analysed by 2-sample t test. Placebo mean 16.6% (s.d.+/- 4.1, n=15) versus GM-CSF mean 17.6% (s.d. +/- 5.7, n=16), p= 0.62.

5.14.12.2 Proportion of regulatory T cells on day 2

There was no significant difference seen in the mean Treg proportion between the two groups on day 2, when analysed by two-sample t-test.

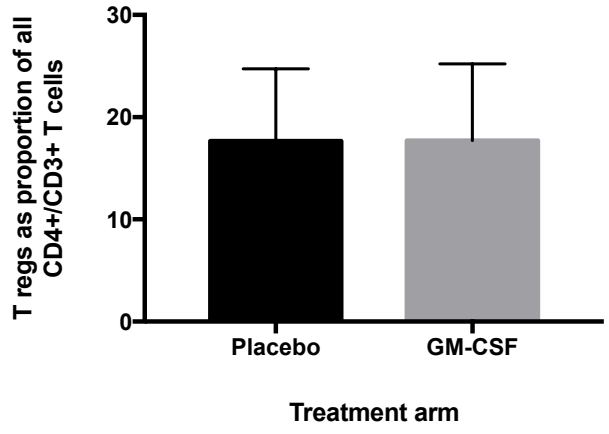

Figure 2.26: Proportion of regulatory T cells (Tregs) on day 2. There was no significant difference seen in the mean Treg proportion between the two groups on day 2, when analysed by two-sample t-test. Mean Treg proportion in the placebo group was 17.7% (s.d. +/- 7.1, range 8-35%, n=14) compared to mean of 17.7% (s.d. +/-7.5, range 5.1-32%, n=11) in the group allocated to GM-CSF, p=0.99.

5.14.12.3 Proportion of regulatory T cells over study course

There was no significant difference in the T reg proportion seen between the two groups on any other study day, when analysed by 2-sample t test. Day 4/5 placebo mean was 18.8% (s.d. +/- 9.2, n=7) compared to GM-CSF 16.9% (s.d. +/- 7.7, n=6). Day 6/7 placebo mean was 19.7% (s.d. +/- 7.7, n=12) compared to 20.5% (s.d. +/- 5.0, n=7). Day 8/9 Placebo mean was 19.1% (s.d. +/- 7.8, n=9) compared to 19.5% (s.d. +/- 5.6, n=6).

5.14.12.4 Differentiation of regulatory T cells

There was no significant difference seen in the differentiation of T cells at baseline or day 2 when analysed by two-sample t-test. Mean proportion of memory cells ($CD45RA^{+}RO^{-}$) at baseline was 35.3% in the placebo group (s.d. +/-12.8, n=17) and 36.5% (s.d. +/- 12.8%, n=15) in the GM-CSF group. On day 2 mean proportion of memory cells was 34.7% in the placebo group (s.d. +/-14.5, n=15) and 35.6% (s.d. +/- 12.2%, n=12) in the GM-CSF group. Mean proportion of naïve cells (CD45RA-RO+) at baseline was 48.8% in the placebo group (s.d. +/-15.3, n=17) and 48.1% (s.d. +/- 12.7%, n=15) in the GM-CSF group. On day 2 mean proportion of naïve cells was 48.3% in the placebo group (s.d. +/-17.5, n=15) and 49.6% (s.d. +/- 13.9%, n=12) in the GM-CSF group

5.15 Per-protocol analyses

The number of patients who triggered study drug termination criteria prior to assessment of the primary outcome was greater than had been anticipated. As described in section 5.10 a total of 5 (5/38, 13.2%) patients received less than 2 doses of study drug prior to assessment of neutrophil phagocytic capacity on day 2 (1 in placebo group, 4 in GM-CSF group). A per protocol analysis was carried out to investigate whether there were differences in primary outcome in the cohort of patients who had received at least 2 doses of GM-CSF or placebo.

5.15.1 Per-protocol analysis neutrophil phagocytic capacity on day 2

There was a significant difference in neutrophil phagocytic capacity seen at day 2 between the 2 groups when analysed per-protocol (p=0.048). This significance was lost when adjusted for baseline neutrophil phagocytic capacity and site (p=0.497).

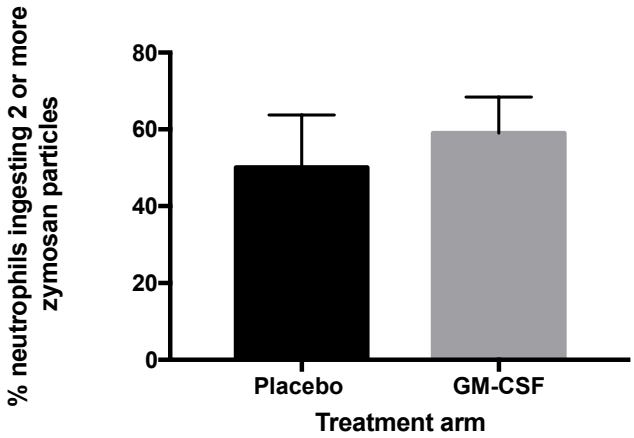

Figure 5.27: Per-protocol analysis of mean neutrophil phagocytic capacity at day 2. A significant difference was seen between the two groups on day 2 when analysed by 2-sample t-test. Mean placebo 50.1% (s.d.+/- 13.7, n= 20), mean GM-CSF 59.1% (s.d.+/- 9.4, n= 13), p=0.048.

Mean neutrophil phagocytic capacity on day 2 in the patients receiving less than 2 doses GM-CSF was 45.3% (s.d. 32.3%, n=2). Results from 2 patients receiving less than 2 doses of GM-CSF were not available on day 2; one patient had died on day 1, and one patient met study drug termination criteria prior to receiving any drug (and was not sampled further following baseline assessment).

5.15.2 Per-protocol analysis of neutrophil phagocytic capacity area under the curve

There was a significant difference between the 2 groups when neutrophil phagocytic capacity area under the curve to day 9 was analysed per-protocol (p=0.011). This significance was lost when adjusted for baseline neutrophil phagocytic capacity and site (p=0.14). The curves were seen to diverge in particular up to day 4/5.

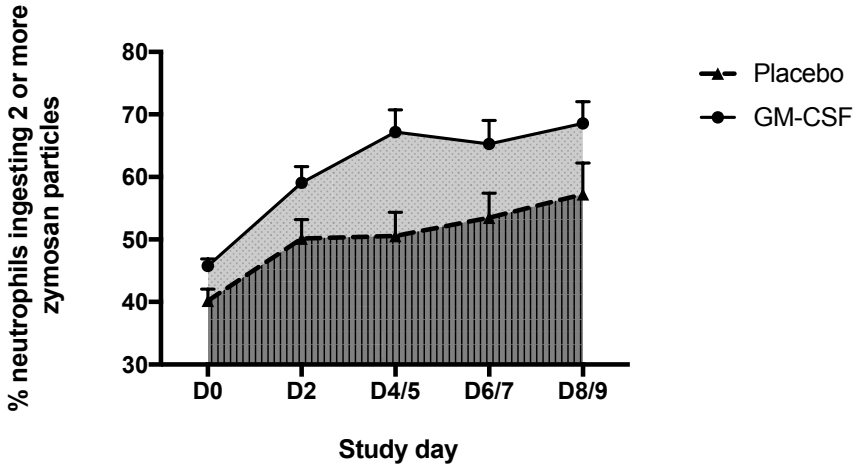

Figure 5.28: Per-protocol analysis of neutrophil phagocytic capacity area under the curve to day 8/9. A significant difference was seen between the two groups when area under the curve to day 9 was analysed by 2-sample t-test. Mean AUC placebo 451.9 (s.d.+/- 85.2, n= 11), mean AUC GM-CSF 553.9 (s.d.+/- 73.5, n= 13), p=0.011.

5.15.3. Per-protocol analysis of proportion of patients with adequate phagocytic capacity on day 2

There was a significant difference seen in the proportion of patients with adequate neutrophil phagocytic capacity, between the two groups, on day 2 when analysed per-protocol for those receiving at least 2 doses of study drug (placebo 45%, GM-CSF 85%, p =0.032).

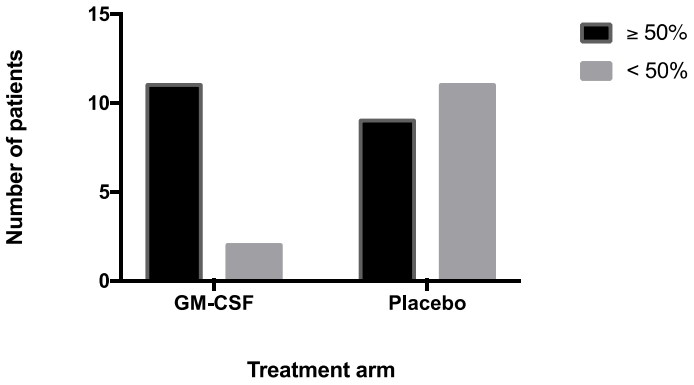

Fig 5.29: Per-protocol analysis of the proportion of patients with neutrophil phagocytic capacity ≥50% or <50%on day 2. There was a significant difference seen between the 2 groups with regard to the proportion of patient with adequate neutrophil phagocytic capacity (i.e. ≥ 50%) on day 2 when analysed per-protocol by Fisher's exact test - placebo =45% (9 of 20 patients) versus GM-CSF 85% (11 of 13 patients), p=0.032.

5.15.3.1 Per-protocol analysis of the proportion of patients with adequate phagocytic capacity on day 4/5, day 6/7, day 8/9

A significant difference was also seen in the proportion of patients, in each group, with adequate neutrophil phagocytic capacity on day 4/5 and day 6/7, when analysing per-protocol for those receiving at least 2 doses of study drug. On day 4/5 46.7% (7 of 15 patients) of the placebo group had adequate phagocytic capacity compared to 90% (9 of 10 patients) of the GM-CSF group, p= 0.04. On day 6/7 46.7% (7 of 15 patients) of the placebo group had adequate phagocytic capacity compared with 100% (9 of 9 patients) in the GM-CSF group, p= 0.009. There was no significant difference seen between the 2 groups on day 8/9 when analysed per-protocol when 63.6% (7 of 11 patients) in the placebo group had adequate phagocytic capacity compared to 100% (8 of 8 patients) in the GM-CSF group, p= 0.103.

5.15.4 Per-protocol analysis of neutrophil superoxide release on day 2

There was no significant difference in neutrophil superoxide release between the two groups on day 2 following administration of the study drug when analysed per-protocol. Mean superoxide release in the placebo group was 1.95 nmol $O_2^-/10^6$ neuts (s.e.m. +/- 0.35, n=16) versus 2.65 nmol $O_2^-/10^6$ neuts (s.e.m, +/- 0.41, n=13) in the GM-CSF group, p=0.20.

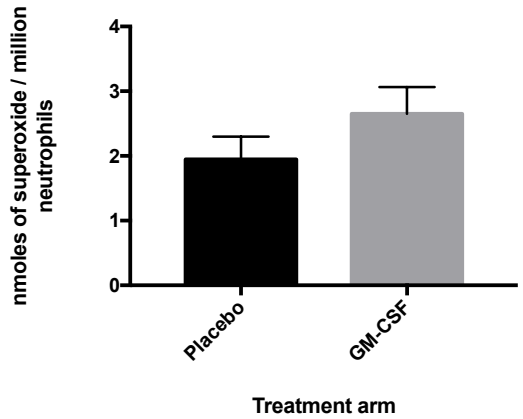

Figure 5.30: Per-protocol analysis of mean neutrophil superoxide release on day 2. There was no significant difference seen between the two groups on day 2 when analysed per-protocol by 2-sample t-test. Placebo mean 1.95 nmol $O_2^-/10^6$ neuts (s.e.m. +/- 0.35, n=16), GM-CSF mean 2.65 nmol $O_2^-/10^6$ neuts (+/- s.e.m. 0.41, n=13), p=0.20.

5.15.5 Per-protocol analysis of neutrophil chemotaxis on day 2

There was no significant difference seen in neutrophil chemotaxis, between the two groups, on day 2 when analysed per-protocol for those receiving at least 2 doses of study drug. Mean migration distance in the placebo group was 381.4 μm (+/- 278.6) compared to a mean of 405.4 μm (+/- 239.2) in the GM-CSF group, p=0.82.

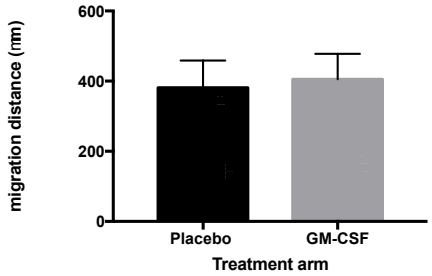

Figure 5.31: Per-protocol analysis of neutrophil chemotaxis on day 2. There was no significant difference seen in neutrophil chemotaxis, between the two groups of patients, on day 2 when analysed per-protocol by 2-sample t-test. Distance migrated on chemotaxis assay (μm) placebo 381.4 (s.d +/- 278.6, n= 10), GM-CSF 405.4 (s.d. +/- 239.2, n=11), p=0.82.

5.15.6 Per-protocol analysis of neutrophil apoptosis on day 2

There was no significant difference seen in neutrophil apoptosis on day 2.

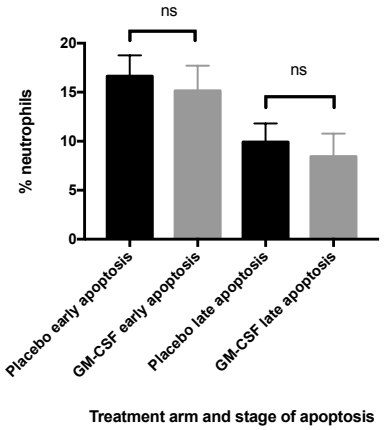

Figure 5.32: Per-protocol analysis of neutrophil apoptosis on day 2. There was no significant difference seen in neutrophil apoptosis, between the two groups of patients, on day 2 when analysed per-protocol by 2-sample t-test. Mean early apoptosis - placebo 16.6% (s.e.m. +/- 2.1, n=16), GM-CSF 15.1% (s.e.m. +/- 2.6, n=13), p= 0.65; mean late apoptosis - placebo 9.9% (s.e.m. +/- 1.9, n=16) GM-CSF 8.4% (s.e.m +/- 2.3, n=13), p= 0.62.

5.16 Clinical Outcomes

5.16.1 Sequential organ failure assessment (SOFA) score

The change in SOFA score between baseline and day 7 was assessed. As the SOFA score represents ordinal data analysis was performed by Mann Whitney test. There was no significant difference in change in SOFA score seen between the two groups, median change in the placebo group was 4 (IQR -0.25 to 8.25) compared to median change in SOFA score in GM-CSF group of 2.5 (IQR 0 to 4.5), p=0.59.

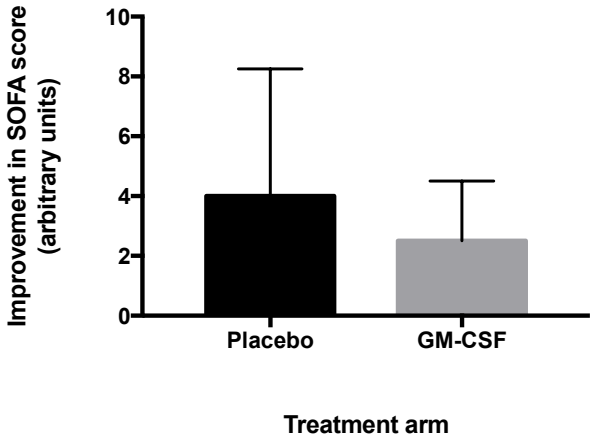

Figure 5.33: Change in sequential organ failure assessment (SOFA) score between day 0 and day 7. There was no significant difference seen in the change in SOFA score from day 0 to day 7 between the two groups when analysed by Mann Whitney U test. Median change in SOFA score in the placebo group 4 (IQR -0.25 to 8.25, n=18) versus median change in the GM-CSF group 2.5 (IQR 0 to 4.5, n=10), p=0.59.

5.16.2 Duration of mechanical ventilation

There was no significant difference seen, between the two groups, in the duration of mechanical ventilation in those patients surviving to 30 days. Median duration of mechanical ventilation in the placebo group was 8 days (IQR 2 to 23) compared to median duration of 9 days in the GM-CSF group (IQR 2.5 to24), p=0.99.

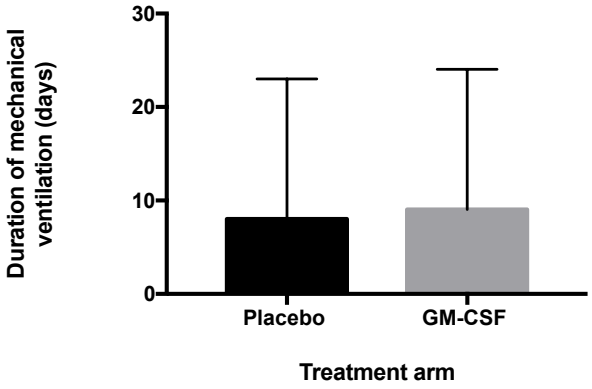

Figure 5.34: Duration of mechanical ventilation in patients surviving to day 30. Median in the placebo group 8 days (IQR 2-23, n=15), compared to median in the GM-CSF group of 9 days (IQR 2.5-24, n=13), p=0.99 Mann Whitney U test.

5.16.3 Duration of ICU stay

There was no significant difference seen, between the two groups, in the duration of ICU stay in those patients surviving to 30 days. Median duration of ICU stay in the placebo group was 17 days (IQR 5 to 30) compared to median duration of 19 days in the GM-CSF group (IQR 7.5-30), p=0.75.

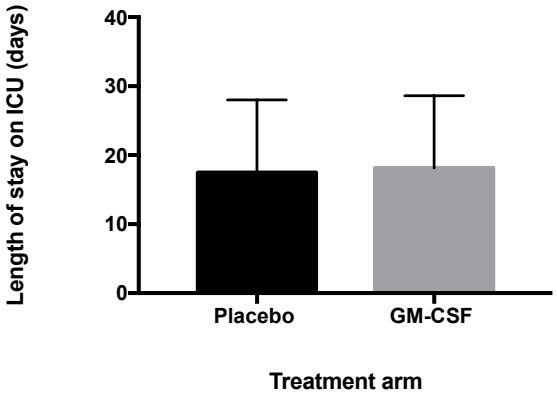

Figure 5.35: Length of ICU stay in patients surviving to day 30. There was no significant difference seen in duration of ICU stay when analysed by Mann Whitney test. Median placebo group 17 (IQR 5-30, n=15), median GM-CSF group 19 (IQR 7.5-30, n=13), p=0.75

5.16.4 Status of patients alive at day 30

There was no significant difference seen, between the two groups, when comparing the status of patients alive at day 30. Of the 15 patients in the placebo group who were alive at day 30, 5 had been discharged home and 10 remained as inpatients (6 on a general ward and 4 on ICU). This compared with the 13 patients in the GM-CSF group where 5 had been discharged home and a further 8 remained as inpatients (4 on a general ward and 4 on ICU).

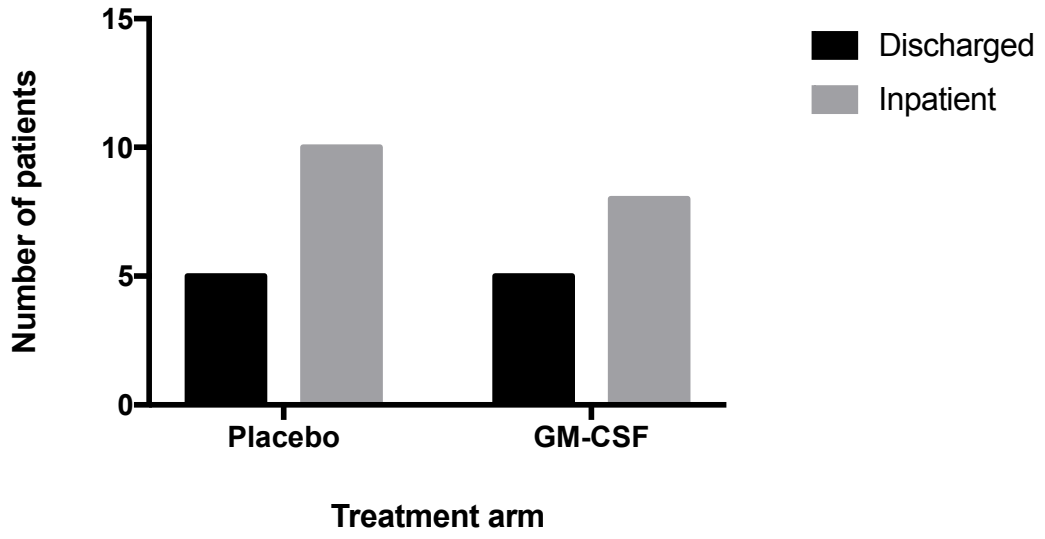

Figure 5.36: Status of patients surviving to day 30. There was no significant difference seen in the status of patients surviving to day 30 when analysed by Chi Squared test. p=0.78

5.16.5 All-cause mortality, day 30

There were 10 deaths during the course of the study up to day 30. 6 deaths occurred in patients in the placebo group (6/21, 28.6%) and 4 in patients in the GM-CSF group (4/17, 23.5%). 9 of the deaths occurred while participants were still an inpatient on ICU. One death occurred following discharge to the ward. When examining deaths in the sub-group of patients who received at least 2 doses of study drug all-cause mortality was 7.7% (1/13) in the GM-CSF group compared to 30% (6/20) in the placebo group.

There was no significant difference in all-cause mortality between the two groups when analysed by log-rank test, p=0.73.

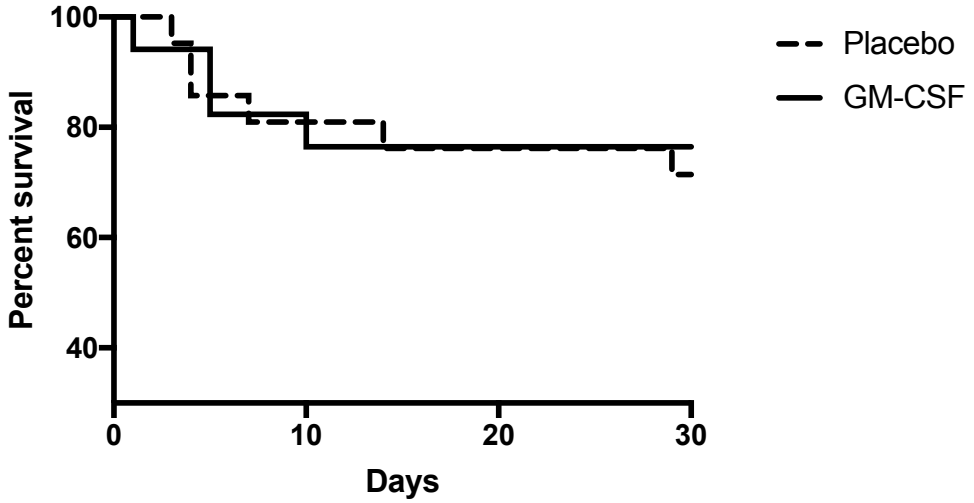

Figure 5.37: Survival curve up to day 30. There was no significant difference in all-cause mortality between the two groups at day 30. Log-rank test, p=0.76.

Treatment allocation	Time from randomisation to death (days)	Cause of death
Placebo	14	Multi-organ failure
Placebo	4	Refractory septic shock
Placebo	7	Sepsis secondary to small bowel perforation
Placebo	29	Long-term post-operative complications of emergency nephrectomy
Placebo	2	Sepsis, stroke, type II respiratory failure
Placebo	3	Pneumonia and cardiac failure
GM-CSF*	10	Pneumonia, multiple comorbidities
GM-CSF**	1	Large bowel obstruction, multiple comorbidities
GM-CSF	5	Chronic obstructive pulmonary disease, type II respiratory failure, acute kidney injury
GM-CSF***	5	Biliary sepsis

Table 28: Cause and timing of death for patients in RCT according to treatment allocation. *, only 1 dose of GM-CSF administered due to transaminitis on day 1; ** only 1 dose administered; *** only 1 dose administered due to thrombocytopaenia.

5.16.5 Incidence of ICUAI

As described in chapter 2 incidence of ICUAI was assessed according to the HELICS criteria (HELICS Protocol 6.1, 2004). Over the course of the study there were 22 confirmed episodes of ICUAI.

Between day 0 and day 9, 13 cases of ICUAI were reported in 10 unique patients. Six cases occurred in the GM-CSF group and 7 in the placebo group.

	Incidence of ICU-acquired infection (ICUAI)									
Arm	D0	D1	D2	D3	D4	D5	D6	D7	D8	D9
GM-CSF	1	0	3	0	2	0	0	0	0	0
Placebo	0	0	2	0	2	0	0	0	2	1

Table 29: Incidence of ICU-AI from study day 0 to study day 9. D, day

From day 10 to day 30 a further 9 cases of ICUAI were reported in 6 patients (3 episodes in 3 patients within the GM-CSF group and 6 episodes in 3 patients within the placebo group). When the data were analysed per protocol according to the number of patients who had received at least 2 doses of GM-CSF/ placebo the number of cases of ICUAI was unchanged.

	Incidence of ICU-acquired infection (ICUAI)						
Arm	D10	D14	D15	D20	D22	D24	D30
GM-CSF		1			1	1	
Placebo	1		2	1			2

Table 30: Incidence of ICU-AI from study day 10 to study day 30. D, day

5.17 Safety data

5.17.1 Blood monitoring

5.17.1.1 White blood cells

GM-CSF was seen to be associated with a rise in total white cell count and a rise in neutrophil count. Both parameters were seen to rise in the GM-CSF group until day 3 following which there was a slight fall before a relatively stable course was followed. Maximum total white cell count seen in any patient was 43.8 $x10^9$/L seen on day 4 in a patient in the GM-CSF group. The corresponding neutrophil count was 36.5 $x10^9$/L. Median total white cell count and neutrophil count remained elevated in the GM-CSF group compared to the placebo group up to day 9. No patient triggered study drug termination criteria on account of their total white cell count.

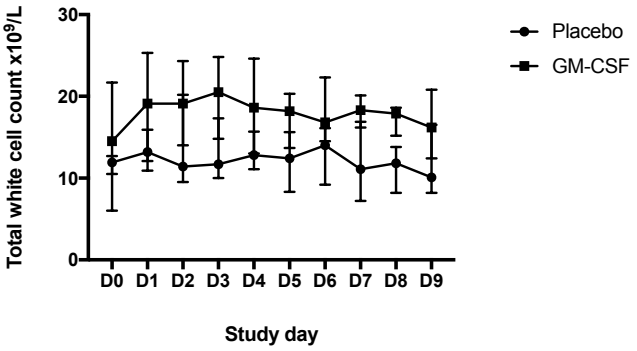

Figure 5.38: Total white cell count over study course.

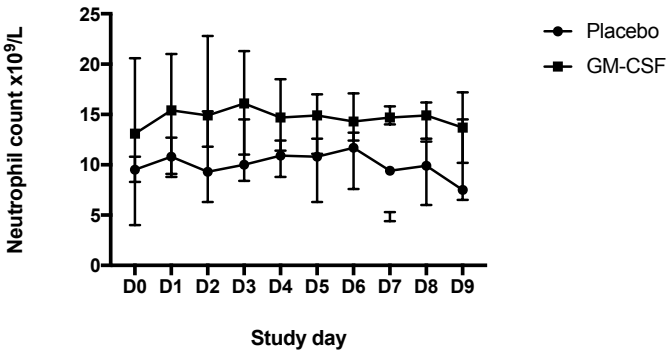

Figure 5.39: Total neutrophil count over study course.

5.17.1.2 Haemoglobin

A gradual fall in haemoglobin level was observed in both groups over the course of the study.

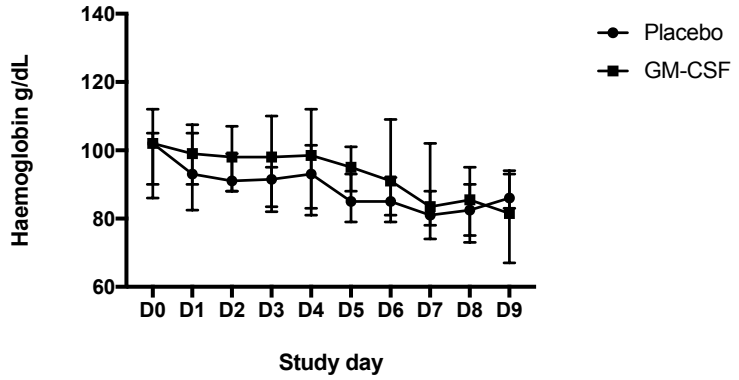

Figure 5.40: Haemoglobin level g/dL over study course.

5.17.1.3 Platelet count

Baseline platelet count was observed to be higher in the GM-CSF group. Platelet count was seen to rise slowly from day 5 in both groups.

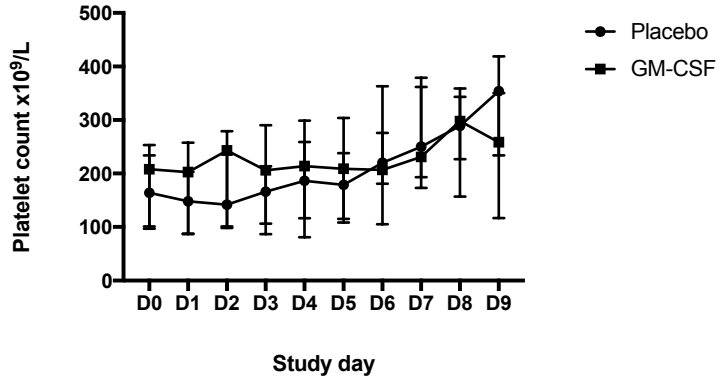

Figure 5.41: Platelet count over study course.

5.17.1.4 Serum creatinine

Baseline creatinine was observed to be higher in the placebo group at baseline, but in both groups serum creatinine appeared to follow a stable course over the10-day sampling period.

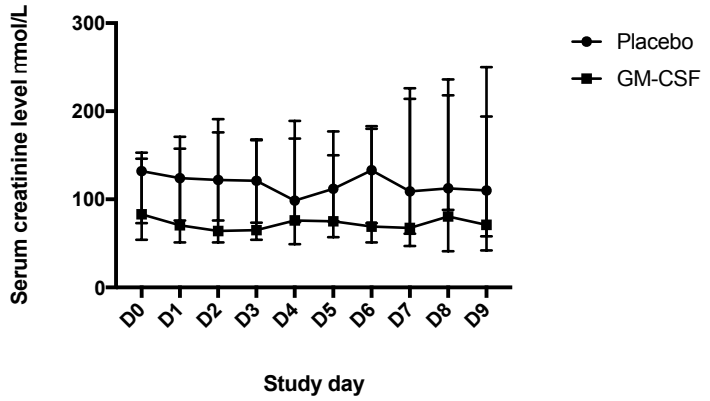

Figure 5.42: Serum creatinine over study course.

5.17.1.5 Liver function tests

Alanine aminotransferase (ALT) levels were similar at baseline between the groups. ALT was seen to rise in the GM-CSF group until day 4 following which it followed a stable course. ALT was seen to follow a stable course in the placebo group. Aspartate aminotransferase levels were seen to follow a stable course in both groups.

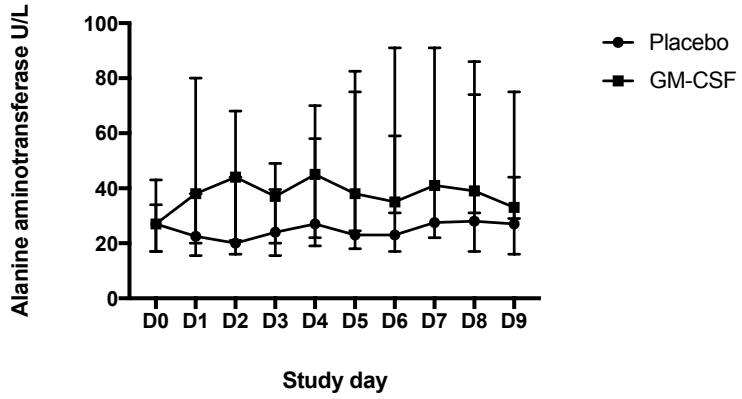

Figure 5.43: Serum alanine aminotransferase over study course.

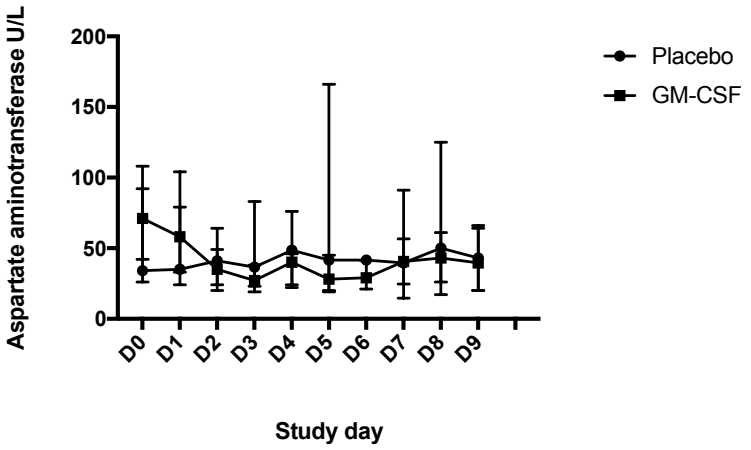

Figure 5.44: Serum aspartate aminotransferase over study course.

5.17.2 PaO_2:FiO_2

As described previously, due to concerns related to the potential for GM-CSF to induce or accelerate acute lung injury, highest and lowest PaO_2:FiO_2 were recorded on a daily basis for each patient whilst on ICU.

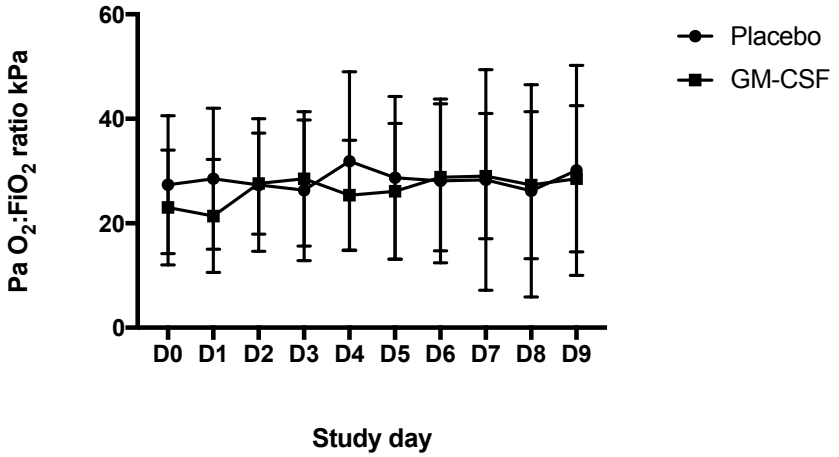

Figure 5.45: PaO_2:FiO_2 over course of study. Mean daily lowest PaO_2:FiO_2 (kPa) over course of study. No significant difference was seen between the lowest recorded PaO2:FiO2 on any study day. P>0.05 on all days. Mean +/- s.d.

Study Day		Placebo		GM-CSF	
		Lowest ratio	Highest ratio	Lowest ratio	Highest ratio
0	n	20	20	16	15
	mean (s.d.)	27.4 (13.2)	38.3 (17.7)	23.0 (11.0)	33.9 (14.9)
	range	(7.6, 54.5)	(11.4, 65.0)	(9.9, 49.5)	(13.2, 59.1)
1	n	20	20	16	16
	mean (s.d.)	28.5 (13.5)	42.5 (19.4)	21.4 (10.8)	38.3 (14.3)
	range	(7.9, 52.8)	(9.2, 91.3)	(8.3, 52.4)	(18.0, 65.5)
2	n	20	20	14	14
	mean (s.d.)	27.3 (12.7)	38.8 (15.6)	27.6 (9.7)	37.0 (15.3)
	range	(6.7, 50.0)	(10.6, 61.4)	(10.9, 43.8)	(16.5, 68.5)
3	n	19	19	13	13
	mean (s.d.)	26.3 (13.5)	44.2 (18.5)	28.5 (12.9)	38.7 (16.7)
	range	(6.4, 51.0)	(10.9, 69.0)	(10.9, 47.6)	(12.6, 65.0)
4	n	19	17	12	12
	mean (s.d.)	31.9 (17.1)	41.5 (21.6)	25.4 (10.5)	37.5 (21.4)
	range	(8.8, 70.8)	(12.2, 92.1)	(9.2, 42.6)	(10.4, 92.1)
5	n	14	14	12	12
	mean (s.d.)	28.7 (15.6)	39.7 (16.5)	26.1 (13.0)	38.5 (16.3)
	range	(8.9, 51.9)	(15.6, 63.5)	(9.0, 50.6)	(20.8, 71.5)
6	n	12	13	10	10
	mean (s.d.)	28.1 (15.7)	39.3 (18.0)	28.8 (14.1)	39.1 (15.8)
	range	(7.4, 52.6)	(11.3, 66.0)	(7.8, 50.2)	(9.9, 63.0)
7	n	10	10	10	10
	mean (s.d.)	28.3 (21.1)	40.1 (31.2)	29.0 (12.0)	37.1 (17.0)
	range	(8.1, 71.1)	(10.4, 92.1)	(9.8, 46.3)	(11.2, 60.9)
8	n	11	11	10	10
	mean (s.d.)	26.2 (20.3)	42.8 (31.3)	27.3 (14.1)	36.5 (14.2)
	range	(6.2, 54.6)	(12.1, 92.1)	(9.0, 50.2)	(12.9, 54.6)
9	n	11	11	9	9
	mean (s.d.)	30.1 (20.1)	40.4 (26.1)	28.5 (14.0)	41.4 (19.5)
	range	(8.8, 60.7)	(11.8, 92.1)	(9.9, 54.6)	(12.4, 64.4)

Table 31: Mean daily PaO2:FiO2 ratio, kPa

There was no significant difference observed in the PaO_2:FiO_2 between the two groups on any study day.

5.17.3 Adverse event reporting

5.17.3.1 Adverse events

Adverse events were classified on their seriousness, their expectedness and their severity. The severity of an adverse event was graded on a three-point scale as mild, moderate or severe.

Study day	No of episodes	GM-CSF			Placebo		
		Mild	Moderate	Severe	Mild	Moderate	Severe
D0	1	0	0	0	1	0	0
D1	4	1	2	0	1	0	0
D2	2	2	0	0	0	0	0
D3	3	3	0	0	0	0	0
D4	1	1	0	0	0	0	0
D5	0	0	0	0	0	0	0
D6	2	0	0	0	1	1	0
D7	1	1	0	0	0	0	0
D8	0	0	0	0	0	0	0
D9	1	1	0	0	0	0	0

Table 32: Adverse events

There were a total of 15 adverse events reported. 12 of these adverse events were classified as mild with a further 3 being classified as moderate. There were no severe adverse events reported.

All 3 moderate adverse events were felt to be unlikely to be related to the study drug. 2 of the moderate adverse events were reported in the GM-CSF group (1 patient developed progressive thrombocytopaenia and 1 patient developed a rise in ALT/AST) both occurring on day 1. In the placebo group one patient developed increased oxygen requirements on day 6 on a background of presumed necrotising pneumonia.

Of the 12 mild adverse events 4 were related to the development of a pyrexia (all within the GM-CSF group), 3 related to changes in platelet count (1 patient within

each group developed thrombocytopaenia, 1 patient in the GM-CSF group developed thrombocytosis), 2 related to the finding of internal jugular vein thrombosis (1 within each group), 2 related to abnormal LFTs (both within the GM-CSF group) and one related to episodes of hypoglycaemia.

In summary within the GM-CSF group there were a total of 11 adverse events; 2 moderate and 9 mild of which 3 were felt to be unlikely to be related and 8 possibly related to the study drug. In the placebo group 1 moderate and 3 mild adverse events were observed; 2 felt to be unrelated and 2 unlikely to be related to the study drug.

5.17.3.2 Serious adverse events

There were a total of 3 serious adverse events reported during the course of the study. One of these events related to a patient in the GM-CSF group occurring on day 1. The patient died following emergency surgery for bowel obstruction and this was thought to be unrelated to the study drug. Of the two serious adverse events occurring in patients within the placebo group, one developed sudden oxygen desaturation on day 3 whilst ventilated and was found to have a pneumomediastinum. This was felt to be unlikely to be related to the study drug. The second patient developed a sudden deterioration with type II respiratory failure requiring treatment with NIV on day 4 following discharge from ICU. This was thought to be due to decompensated obstructive sleep apnoea (OSA) but was reported as being possibly related to the study drug.

5.17.3.3 Suspected unexpected serious adverse events

There were no SUSARs reported during the course of the study in either group.

5.18 Discussion

The randomised controlled trial was designed to test the hypothesis that GM-CSF would restore effective neutrophil phagocytosis in critically ill patients with confirmed neutrophil dysfunction. In addition, it aimed to explore the effect of subcutaneous GM-CSF on other elements of neutrophil function, neutrophil receptor expression, monocyte HLA-DR expression and various clinical outcomes (especially safety).

5.18.1 Effect of subcutaneous GM-SCF on neutrophil phagocytic function

Compared to normal saline placebo GM-CSF was not seen to produce a statistically significant difference in neutrophil phagocytic capacity on day 2 following daily sub-cutaneous administration. On examining sequential neutrophil phagocytic capacity on day 4/5 there was a statistically significant difference seen between the two groups, however, this effect was lost when corrected for baseline neutrophil phagocytic capacity and site. No significant difference was seen on day 6/7 or day 8/9. The statistically significant difference in the area under the curve for neutrophil phagocytic capacity up to day 9 and the divergence of the curves in particular up to day 4/5 does suggest some evidence for a biological effect of GM-CSF on neutrophil phagocytic function. However again this effect was lost when correcting for baseline neutrophil phagocytic capacity and site. The unexpected and statistically significant difference in baseline neutrophil phagocytic capacity between the two groups is likely to have had a significant impact on the power of the study to demonstrate an effect in a cohort of this size. All significant results have been adjusted for baseline phagocytic capacity and site. In addition, during the RCT, the placebo group of patients was observed to have a natural recovery of neutrophil function over time. This recovery was greater than we had anticipated (chapter 2, section 2.18.3, page 83). As discussed in chapter 4 there have not, to my knowledge, been any published studies examining the natural course of recovery in neutrophil function during critical illness and this study provides some evidence to show that neutrophil recovery does occur to some degree even during these relatively early stages of critical illness. However, despite this, other evidence of immune dysfunction is seen to persist. With this new observation applied to our previous calculations a larger cohort of patients would be required to power the study to demonstrate a biological effect of GM-CSF.

5.18.2 Timing of assessment of primary outcome

The choice of timing of assessment of the primary outcome may arguably have been more appropriately placed on day 4 following administration of all 4 doses of the study drug when the maximal biological effect could have been evaluated. The rationale for our original decision regarding assessment of the primary outcome on day 2 was that, in view of the fact that ICUAI is defined as any infection occurring after 48 hours of admission to ICU (World Health Organization., 2002) we hoped that we could demonstrate a potentially protective biological effect of GM-CSF within this time window. It is likely however that the maximal impact of GM-CSF had not yet been achieved and that the power of the study to demonstrate benefit was weakened as a result.

5.18.3 Adequacy of primary outcome in assessing the biological effect under investigation

As we were investigating the biological effect of GM-CSF on neutrophil function, as a proof of concept study to inform a larger trial to assess whether GM-CSF may reduce ICU-acquired infection, the impact on the proportion of patients having 'adequate neutrophil function' may have had greater relevance. As discussed, previous work has shown that a neutrophil phagocytic capacity < 50% is associated with an increased risk of ICUAI (Conway Morris et al 2009) and that neutrophil phagocytic capacity ≥50% may confer some form of protection. The fact that a statistically significant difference was seen in the proportion of patients with adequate neutrophil function in the GM-CSF group on all study days up to and including day 6/7 could be of greater clinical importance than the mean difference in absolute phagocytic capacity on each those days. On reflection our choice of primary outcome may not enable the most sensitive or rigorous assessment of the potential for GM-CSF to affect neutrophil function in a manner that would afford protection against ICUAI.

The observation that by day 8/9 the significant difference seen in the proportion of patients with adequate neutrophil function had been lost may suggest that GM-CSF speeds the rate of recovery of neutrophil phagocytic capacity while over time the natural rate of recovery seen in the placebo group catches up. As ICUAI can develop at any time after 48 hours of admission to the ICU (World Health Organization., 2002) the ability to restore adequate neutrophil function earlier in the course of a

patient's admission may potentially offer significant clinical benefit with improvement in outcomes related to both morbidity and mortality.

5.18.4 Loss of follow up of laboratory data due to patient recovery or death

Unfortunately, due to the logistics of the trial, in some cases it was not possible to collect blood samples from patients for analysis of neutrophil functional outcomes after they had been discharged from ICU to another location within the hospital (n=2). Collection of blood samples was not continued after a patient had been discharged from hospital during the sampling follow-up period day 0 to day 9 (n=3). In addition, the study cohort was affected by the deaths of seven patients (7/38,18.4%) between day 0 and day 9, further diminishing the sample size in both groups. This unavoidable loss in potential data on each sampling day during the RCT will undoubtedly have had an effect on the power of the study as the numbers remaining in each group, in particular the GM-CSF group, at day 2 fell below the number required to ensure the power of the study to assess the primary outcome (n=17, chapter 2, section 2.18.3). In addition, there is the potential for these data omissions to have skewed the remaining data. It could be postulated that patients with an increased severity of critical illness may be both at higher risk of death and have a poorer rate of recovery of their neutrophil phagocytic capacity up to the point of their death (negatively skewed results) while patients with a lower severity of critical illness may follow a rapid trajectory to recovery, leading to discharge from the ICU, and have a greater rate of recovery of neutrophil phagocytic capacity following discharge from ICU (positively skewed results) reflecting their clinical improvement. Data for 3 patients were lost from the GM-CSF group due to recovery (compared to 1 from the placebo group), while data from 3 patients were lost from the GM-CSF group due to death (compared to 4 from the placebo group). Given the small sample size the potential exists for the remaining data to be skewed by the absence of these data.

5.18.5 Per-protocol analysis of data

There was a greater than expected number of patients triggering study drug termination criteria prior to receiving 2 doses of GM-CSF/placebo, which was the number of doses intended to be administered prior to assessment of the primary outcome. In addition, there was an imbalance between the groups in those patients affected, with a greater number of patients triggering study drug termination criteria within the GM-CSF group. When a planned per-protocol analysis was carried out a statistically significant difference was seen in the primary outcome, however this effect was lost when adjusted for the unexpected difference in baseline neutrophil phagocytic capacity. As with the intention-to-treat analysis there was a significant difference in the area under the curve up to day 8/9, with divergence of the curves up to day 4/5. However, once again the significance was lost when adjusted for baseline differences. The impact of these missing patients on both intention-to-treat and per-protocol analysis will have affected the power of the study.

5.18.6 Effect of subcutaneous GM-CSF on monocyte HLA-DR expression

In keeping with the DFS it was once again observed that GM-CSF administration was associated with a significant rise in the levels of mHLA-DR expression, but that this effect was lost shortly after all 4 doses had been completed. Extensive evidence exists to demonstrate that mHLA-DR is a reliable marker of immunosuppression and immunoparesis (Monneret et al., 2006, Cheron et al., 2010,) and that restoration of HLA-DR expression on the surface of monocytes has been associated with faster recovery from critical illness (Cheron et al., 2010, Tschaikowsky et al, 2006).The fall in mHLA-DR expression on cessation of the daily GM-CSF injections may indicate that the duration of therapy was inadequate to sustain the response to GM-CSF and that the risk of ICUAI may increase again at this point. Previous studies of the effect of GM-CSF on mHLA-DR expression have not made any attempt to quantify whether the restoration of mHLA-DR levels to the healthy range is associated with any improvement in the functional capacity of monocytes in particular with respect to their contribution to immunocompetence. Meisel's study (Meisel et al., 2009) previously showed that when GM-CSF was administered for 10 days the effects in terms of recovery of mHLA-DR persisted over this time course. They did not,

however, carry out any analysis of the course of mHLA-DR expression once the drug had been stopped.

5.18.7 Effect of subcutaneous GM-CSF on neutrophil CD88 expression

GM-CSF was not seen to have any effect on neutrophil CD88 expression over the course of the study. In addition, there was no correlation seen between neutrophil phagocytic capacity and neutrophil CD88 expression at baseline in the RCT. This supported our previous decision not to use neutrophil CD88 expression as a surrogate marker for phagocytic capacity when determining final eligibility for entry into the study.

As a significant correlation has previously been identified between neutrophil phagocytic capacity and neutrophil CD88 expression (Conway Morris et al.,2009, Conway Morris et al., 2011), and as we ourselves demonstrated a moderate correlation within the cohorts of patients studied in the ACS (chapter 3, section 3.6), and DFS (chapter 4, section 4.13.3) , we felt that further validation of the possible role of CD88 expression as a surrogate marker for phagocytosis was warranted during the RCT. In our hands, there is no consistent evidence to confirm a significant correlation between these two parameters.

5.18.8 Effect of subcutaneous GM-CSF on other elements of neutrophil function

Previous studies have shown GM-CSF to have an effect on other neutrophil functions including; enhancing neutrophil superoxide release when administered both *ex-vivo* (Lopez et al., 1986, Cebon et al., 1990, Weisbart et al., 1987) and *in-vivo* (Presneill et al., 2002); delaying neutrophil apoptosis (Lopez et al., 1986, Brach et al., 1992) and reducing neutrophil chemotaxis (Lieschke et al., 1992). Within this patient cohort, when administered at this dose, GM-CSF was not seen to have any effect on neutrophil superoxide release, apoptosis or chemotaxis when assessed by either intention-to-treat or per-protocol analysis at any time over the course of the study.

5.18.9 Effect of subcutaneous GM-CSF on regulatory T cell proportions

As has been observed in previous studies of sepsis and critical illness an increased proportion of regulatory T cells, as a proportion of CD4+ cells, was seen in our cohort of patients (Monneret et al.,2003, Venet et al., 2009). In a healthy population the proportion of Tregs is usually between 5-10% (Tatura et al., 2012) while in our group at baseline overall mean was 17.1% Similar proportions, and indeed higher levels, have been seen in previously studied cohorts of patients with a rise in the proportion of Tregs being seen within the first few days of sepsis onset (Monneret et al., 2003, Venet et al., 2009). Mean Treg proportion continued to be elevated in both groups at day 9 and high levels at this stage in the course of illness have been associated with poor survival however out study was not powered to examine this.

5.18.10 Effect of subcutaneous GM-CSF on clinical outcomes

The study was not powered to examine the secondary clinical outcomes and there was no significant difference seen between the two groups in any of the secondary clinical endpoints including length of stay on ICU and in hospital, duration of mechanical ventilation, change in SOFA score and change in PaO_2:FiO_2. Analysis of these outcomes would need to be studied in a much larger cohort of patients to look for differences in outcome. These outcomes are, however, those which are of most importance to patients and the clinical teams responsible for their care.

5.18.11 Effect of subcutaneous GM-CSF on patient outcome

As with other clinical outcomes this study was not powered to look at differences in patient outcome in terms of mortality. Overall ICU mortality rate was 23.7% (9/38) while in hospital mortality rate was 26.3% (10/38). This mortality rate is in the expected range for this cohort of critically ill patients where median APACHE II score was 20 (19.5 GM-CSF group, 21 placebo group). Predicted in hospital mortality for a patient with an APACHE II score of 20 admitted to ICU with sepsis is 38.1%, rising to 50% for a patient admitted with sepsis post emergency surgery. Although mortality rates were similar in each group when analysed on an intention to treat basis the absolute difference in mortality rate seen in those patients receiving at least 2 doses of study drug was 22% (8%(1/13) GM-CSF group compared to 30%(6/20) placebo group).There was no difference seen in the status of patients surviving to day 30 with

33% of the placebo group and 38% of the GM-CSF group having been discharged home while the rest of patients in each group remained inpatients within the hospital with similar numbers in each group still on ICU.

5.18.12 Observed safety profile of subcutaneous GM-CSF

Administered at this dose and for this duration subcutaneous GM-CSF appears to be safe when administered to critically ill patients. As expected GM-CSF was seen to be associated with a rise in total white cell count and a rise in neutrophil count with median total white cell count and neutrophil count remaining elevated in the GM-CSF group compared to the placebo group up to day 9. No patient developed a rise in total white blood cell count such that study drug termination criteria were triggered and no complications were seen in relation to leucocytosis. The gradual fall in haemoglobin level observed was similar in both groups and is in keeping with the observed response to critical illness (Nguyen et al., 2003). Prior to commencing the study, we discussed the impact of the trial blood sampling procedures with an independent haematologist who felt that there was no risk of inducing anaemia as a consequence of the sample volumes collected. Baseline platelet count was observed to be higher in the GM-CSF group with platelet count being seen to rise slowly from day 5 in both groups. This may have reflected some degree of recovery from the critical illness insult (Hui et al., 2011). There have been previous reports of elevations in serum creatinine following administration of GM-CSF (Sanofi-Aventis, 2013). Alternate week serum monitoring is advised within the product SmPC suggesting that this may be a longer term effect of GM-CSF administration. Baseline creatinine was observed to be higher in the placebo group but in both groups serum creatinine appeared to follow a stable course over the10 day sampling period. Elevations in liver enzymes have also been reported previously in patients following administration of GM-CSF (Leischke et al., 1992, Honkoop et al., 1996). Alanine aminotransferase (ALT) levels were similar at baseline between the groups however, ALT was seen to rise in the GM-CSF group until day 4 following which it followed a stable course. Despite the rise in ALT median levels remained within the normal range. ALT was seen to follow a stable course in the placebo group. Aspartate aminotransferase levels were seen to follow a stable course in both groups.

With respect to adverse events there were an acceptable number of adverse events reported none of which were deemed to be severe in nature. Of the 3 adverse events of moderate severity all were considered to be unlikely to be related to the study drug. Although there was a greater number of adverse events seen in the GM-CSF group (11) versus the placebo group (4) the majority of these were considered to be mild. Of the three serious adverse events reported during the study only one of these was thought to be possibly related to the study drug. Following unblinding it was confirmed that this patient had received placebo.

As had been observed in the DFS there was no difference seen in the PaO2:FiO2 following administration of GM-CSF. Despite the theorectical concerns and previously reported data (Goodman et al., 1999) regarding the risk of ALI with GM-CSF our results add to the more recent data suggesting that GM-CSF is not associated with an increased risk of ALI (Presneill et al., 2002, Paine et al., 2012).

The favourable safety profile of GM-CSF observed suggests that in future studies our strict exclusion criteria may be able to be relaxed to enable recruitment of a broader range of patients who may potentially benefit from the drug.

5.18.13 Blinding issues

As discussed previously there were significant logistical challenges in implementing blinding for the study. These were overcome by identifying unblinded research nurses to be involved in the reconstitution and administration of the study drug at all research sites. As discussed in section 5.13, at two sites a possible breach of blinding was retrospectively identified during the processes of both internal and external audit. The potential breach related to research nurses having been involved in the transposition of clinical data from source data to the e-CRF in patients to whom they had administered IMP. Concerns were raised that this could have led to bias within these data. There was no evidence for a breach of blinding at any other study site or in any other trial process or procedure. As discussed in section 5.13 all clinical data were recorded independently at source, by the clinical nursing team or hospital laboratory as part of usual clinical care. We therefore believe that the potential for bias (through the participation of an unblinded member of the research nursing team in the transposition of clinical data) is considered to be negligible but could not be fully excluded. There was no evidence to suggest that this potential

breach could have had any effect on the research laboratory outcomes which were analysed by blinded staff in a research laboratory location which was geographically distinct from the clinical areas where the administration of study drug and the transfer of clinical data took place. As a consequence of this potential breach, however, the study has been reported as a single-blind study.

5.19 Limitations of the study

5.19.1 Effect of sample size on the power of the study

As has been discussed the power of this study was limited by the unexpected imbalance in neutrophil phagocytic capacity at baseline and an unexpected rate of study drug termination prior to assessment of the primary endpoint. Therefore, while the study provided some evidence to suggest a biological effect of GM-CSF on neutrophil phagocytic capacity the power of the study to confirm this effect may have been lost. This was also impacted upon to some degree by the imbalance in the two allocation groups following randomisation which arose as a result of the randomisation occurring in permutable blocks of variable length, stratified by site. This degree of complexity in the randomisation schedule was designed to minimise the risk of breach of concealment but ultimately led to an imbalance between the groups, given the small sample size.

5.19.2 Uncertainty regarding optimum dose and duration of GM-CSF

Although the dose finding study served to confirm that GM-CSF, at a dose of 3μgm/kg/day for 4 days, appeared to be safe and to be associated with the desired biological effect in terms of improvement in neutrophil function, the lack of comparison treatment arms meant that it did not answer the question as to the optimum dose and duration of GM-CSF. An increase in both the dose and duration of GM-CSF used in the RCT may have resulted in a more significant improvement in neutrophil phagocytic capacity in the treated group. Analysis of the area under the curve data shows an increased rate of rise in neutrophil phagocytic capacity between day 0 and day 4/5 in the GM-CSF group with divergence of the curves up to this

point. Between day 4/5 and day 6/7 mean neutrophil phagocytic capacity is seen to fall slightly in the GM-CSF group before rising again at a slower rate in parallel with the placebo group. This may suggest that after completion of the administration of GM-CSF on day 3 the beneficial effect of GM-CSF on phagocytic function was lost and from that point the natural rate of recovery, seen in the placebo group, was counting for the slower rate of rise in each group. In addition, the data showing that GM-CSF increased mHLA-DR expression to immunocompetent levels initially, followed by a fall to levels associated with immunosuppression on cessation of the drug, do raise the question of whether short duration GM-CSF was inadequate to ensure a sustained response. In addition, it is recognised that the bioavailability of many drugs (in particular subcutaneously administered drugs) is reduced by a variety of factors affecting critically illness (Smith et al., 2012, Dorffler-Melly et al., 2002). Within our patient cohort a significant number of patients received treatment with vasopressor drugs or CVVH during the study drug administration period and both of these factors may have had a significant affect on the bioavailability of the study drug.

5.19.3 Lack of prior evidence regarding the natural course of recovery of neutrophil phagocytic capacity in critical illness

The lack of prior available evidence regarding the natural course of recovery of impaired neutrophil phagocytic capacity in critical illness led to the unexpected finding of a higher rate of recovery in neutrophil phagocytic function among patients randomised to receive placebo than we had anticipated. The impact of this on our power calculation may have led to the study being underpowered to demonstrate a beneficial effect of GM-CSF over placebo. The results in the placebo group within this study are, to our knowledge, the first results to show that recovery in terms of neutrophil phagocytic capacity begins fairly early in the course of the critical illness. Further study in this area is required. Interventions to speed up the rate of recovery may still be of significant benefit but an alternative approach to measuring this may be required.

5.19.4 Effect of logistical challenges on the delivery of the study

This study was recruiting critically ill patients early in the course of their admission to ICU and consequently recruitment was both unpredictable and time critical. In addition, the intervention was required to be delivered seven days a week, necessitating availability of research staff on all days including weekends and bank holidays. For a non-commercial clinical trial operating with limited resources in terms of staffing and finance, timely and successful completion of the trial was a significant challenge. Current local clinical research networks are not set up to enable the sharing of research staff and resources across multiple clinical research sites, and seeking to attain this in the future is likely to contribute to the improved successful completion of similar studies.

5.19.5 Effect of blinding mechanisms on the study

The logistical issues outlined in terms of delivering the trial successfully and in a timely manner also influenced the mechanisms for blinding of this study. As discussed this unfortunately led to the discovery (after the trial had completed) of a potential breach of blinding at two sites. Although we feel confident that no breach of blinding actually occurred and that the potential for bias is negligible we have been unable to publish the study as a double blinded trial and this will undoubtedly have an effect on the scientific impact of the trial and may negatively affect future research in this area.

5.19.6 Effect of sample size on the analysis of clinical outcomes

The study was not powered to assess the effect of GM-CSF on any of the clinical outcomes and results in relation to clinical outcomes are provided by descriptive statistics only. While the biological effects of drugs determine their efficacy the impact on clinical outcomes are of most importance to patients and clinicians. Much larger studies would be required to explore these potential clinical effects in more detail in particular the potential for GM-CSF to prevent the development of ICUAI.

5.20 Future work

Future work in this area should focus on establishing robust pharmacokinetic and pharmacodynamic data in a larger cohort of critically ill patients to establish the optimum dose and duration of GM-CSF which might lead to a beneficial response in terms of neutrophil phagocytic function. Once the optimum dose and duration has been established a large scale multi-centre trial would be required to answer both the biological question of whether or not GM-CSF can speed the rate of recovery of neutrophil phagocytic capacity and the clinical question of whether or not, therefore, GM-CSF could have an impact on the development of ICUAI in such patients. In order to adequately power such studies a deeper understanding of the natural course of neutrophil phagocytic dysfunction in critically ill patients is required. The importance of mHLA-DR expression as a biomarker of immune dysfunction has already been established and this study adds to the data which show that administration of GM-CSF can restore mHLA-DR expression to normal levels. Further work exploring the functional impact of both low mHLA-DR expression and restoration to normal levels following administration of GM-CSF would provide valuable information in terms of further developing targeted therapy

5.21 Conclusion

The results of this study suggest that low dose, short duration treatment with GM-CSF has a small but real biological effect in restoring neutrophil phagocytic capacity in critically ill patients. To our knowledge this is the first demonstration of such an effect in adult critically ill patients with confirmed neutrophil dysfunction. While many previous similar studies have focused on immune cell dysfunction in patients with sepsis we sought to include patients with a wide range of critical illness insults in our population demonstrating the potential relevance across the ICU population. We acknowledge that no single factor is responsible for the immunosuppressed state seen to affect patients during critical illness, and in targeting neutrophil phagocytic function were attempting to impact on one crucial element of host defence in the search for improved clinical outcomes. It is increasingly recognised that the future for improvements in patient care needs to be focussed on delivering personalised medicine. By stratifying patients on the basis of their baseline neutrophil function and targeting those at increased risk this study was seeking to deliver exactly that. To deliver personalised medicine it must be accepted that large numbers of patients may need to be screened in order to identify the target population for a particular drug. While in the past a high screening to recruitment ratio has been viewed as a negative finding (suggesting that the results of studies may not be applicable to the broad population) in the modern era of personalised medicine this will become an expected task. The key to the delivery of such targeted medical care will therefore be to ensure the development of rapid diagnostic screening tests to enable prompt delivery of therapy where it is indicated.

The results of the study suggest that further work is needed to establish whether (a) an increased dose and or duration of GM-CSF might produce a greater biological effect while maintaining the favourable safety profile seen at this dose, and (b) whether ultimately GM-CSF could be effective in reducing the risk of ICUAI in critically ill patients with confirmed neutrophil dysfunction.

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Appendix

Study Protocol

DOES GM-CSF RESTORE EFFECTIVE NEUTROPHIL FUNCTION IN CRITICALLY ILL PATIENTS?

Sponsor	Newcastle upon Tyne Hospitals NHS Foundation Trust
Sponsor Protocol number:	AJSEB001
EudraCT Number:	2011-005815-10
ISRCTN:	
Funder	Medical Research Council
Funding Reference Number	G1100233
Chief Investigator	Prof John Simpson Professor of Respiratory Medicine Institute of Cellular Medicine 4th Floor, William Leech Building Medical School, Newcastle University Framlington Place Newcastle upon Tyne NE2 4HH
REC Number	12/YH/0083
Version Number and Date	Version 8.0, 9th October 2013

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Abbreviation	Full Wording
AE	Adverse event
ANC	Absolute neutrophil count
APACHE	Acute Physiology and Chronic Health Evaluation
BP	Blood pressure
CI	Chief investigator
CRF	Case report form
CTU	Clinical Trials Unit
dL	Decilitre
DMSC	Data Monitoring and Safety Committee
ECG	Electrocardiograph
ELISA	Enzyme-linked immunosorbent assay
FBC	Full blood count
FiO_2	Inspired oxygen fraction
g	Gram
GCP	Good Clinical Practice
GMP	Good Manufacturing Practice
HAI	Hospital-acquired infection
HIV	Human immunodeficiency virus
HR	Heart rate
ICU	Intensive care unit
ICUAI	Intensive care unit-acquired infection
IMP	Investigational Medicinal Product
ISF	Investigator site file
kg	Kilogram
L	Litre(s)
LFTs	Liver function tests
μg	Microgram
mg	Milligram
mL	Millilitres
mmHg	Millimetres of mercury
MHRA	Medicines and Healthcare Products Regulatory Agency
MV	Mechanical ventilation
NCTU	Newcastle Clinical Trials Unit
NIHR	National Institute for Health Research
PaO_2	Partial pressure of oxygen in arterial blood
PerLR	Personal legal representative
PI	Principal investigator
ProfLR	Professional legal representative
R&D	Research and Development
RCT	Randomised controlled trial
REC	Research Ethics Committee
rhu GM-CSF	Recombinant human Granulocyte macrophage colony stimulating factor
ROS	Reactive oxygen species
SAE	Serious adverse event
SaO_2	Oxygen saturation in arterial blood
S/C	Subcutaneous
SIRS	Systemic inflammatory response syndrome
SMF	Study master file
SOFA	Sequential organ failure assessment
SOP	Standard operating procedure
SmPC	Summary of product characteristics
SUSAR	Suspected unexpected serious adverse reaction
U&Es	Urea and Electroytes
UK	United Kingdom
WCC	White cell count

1. STUDY SUMMARY

1.1 Lay Summary

Despite the introduction of multiple preventative measures rates of hospital-acquired infection (HAI) in the intensive care unit (ICU) remain high. New approaches to tackling this problem are required. The neutrophil (a type of white blood cell) is the key cell fighting bacterial and fungal infection in the body. This study group has shown that the majority of patients on intensive care have neutrophils which don't eat germs effectively and are therefore less able to fight off infection. These patients, whose white blood cells don't work properly in this way, are much more likely to develop a second infection whilst in hospital (HAI). These patients can be identified by measuring the levels of a specific receptor on the surface of the neutrophils by a simple blood test.

Previous work carried out by this research group has also shown that adding a drug called granulocyte macrophage-colony stimulating factor (GM-CSF) to a sample of blood from such patients in the laboratory, it is possible to restore the ability of the neutrophils to eat bacteria and fight infection.

This study, therefore, will test whether it is possible to restore the eating ability of critically ill patients' white blood cells, in real life, by giving them GM-CSF as an injection while they are on intensive care.

The study will involve 2 distinct components. The first component will aim to establish the optimal dose of GM-CSF that should be administered in order to improve the function of neutrophils in critically ill patients. Patients with faulty neutrophils who are enrolled into this part of the study will receive a dose of either 3 or 6 microgram/kilogram/day of GM-CSF for 4 or 7 days. We will measure the eating capacity of their neutrophils before and after the injections to see which dose is the most effective in improving their function. At the same time we will monitor the patients' blood tests and clinical condition to look for any unwanted side effects of treatment. The optimal dose (i.e. the one which produces the greatest benefit without significant side effects) will be selected for use in the Randomised Controlled Trial (RCT) that will follow.

The second component of the study, the RCT, will again enrol patients on intensive care whose white blood cells don't work properly in this way. Patients who take part in this component of the study will either receive an injection of the drug (GM-CSF) or an injection of a solution which will have no effect (placebo or dummy drug). We will then compare whether those patients who have received the GM-CSF injection have an improvement in the function of their neutrophils compared to those who don't.

As well as looking at whether or not the white blood cells work properly we will also study whether there is a difference in the rates of infection picked up in hospital between the two groups and also whether there is any difference in their clinical outcomes e.g. length of stay in hospital, time on a ventilator and survival.

If the RCT demonstrates a clear effect for GM-CSF in improving the function of patients' neutrophils, the way would be paved for future studies determining whether GM-CSF can prevent HAI in future, larger studies. Currently, no good drug treatments preventing HAI in the ICU are available.

1.2 Professional Summary

Despite the introduction of multiple preventative measures, nosocomial infection rates remain unacceptably high, particularly in the ICU where 20-40% of patients acquire new nosocomial infections. Novel strategies are therefore urgently required. The neutrophil is the key cellular effector for clearance of bacterial and fungal pathogens. We have demonstrated that: impaired neutrophil phagocytosis is common in ICU patients; patients with impaired neutrophil phagocytosis in ICU are at significantly increased risk of nosocomial infection; and granulocyte macrophage – colony stimulating factor (GM-CSF) applied to patients' impaired neutrophils *ex vivo* restores effective phagocytosis. If subcutaneous (s/c) GM-CSF, targeted to high risk patients with proven neutrophil dysfunction, also restored effective phagocytosis, GM-CSF would be well positioned for comprehensive assessment and development as a novel measure to prevent nosocomial infection in the ICU. GM-CSF has never been evaluated as a therapy specifically targeted to critically ill patients with neutrophil dysfunction in the ICU.

We therefore propose a) to initially carry out a dose finding study to determine the optimal dose/duration of GM-CSF in this specific setting in order b) to perform the first proof of concept, double blind randomised controlled trial (RCT) of s/c GM-CSF specifically targeting critically ill patients with proven neutrophil dysfunction, and therefore at high risk of nosocomial infection. The primary endpoint will be neutrophil phagocyte capacity. A proven beneficial effect for GM-CSF would rapidly pave the way for larger studies assessing its capacity to prevent nosocomial infections in ICU.

1.3 Hypothesis

GM-CSF targeted to critically ill patients with known neutrophil dysfunction restores effective neutrophil phagocytosis.

1.4 Study Aims and Objectives

To test whether clinical administration of GM-CSF replicates the effects seen *ex vivo*, in the blood of critically ill patients, by restoring neutrophil phagocytosis.

1.5 Patient Population

Critically ill patients with evidence of impaired neutrophil phagocytosis. Levels of neutrophil surface CD88 expression correlate with phagocytic capacity and will be used to identify such patients.

1.6 Study Setting

General adult ICUs (Royal Victoria Infirmary (RVI) and Freeman Hospital (FRH), Newcastle upon Tyne, Queen Elizabeth Hospital (QEH), Gateshead) and Sunderland Royal Hospital (SRH).

1.7 Study Intervention

1.7.1 Dose-finding Study

Leukine (Sargramostim, recombinant, yeast derived GM-CSF) 3µg/kg/day OR 6µg/kg/day, administered subcutaneously, for 4 OR 7 days.

Patient Enrolment Number	Dose of Leukine (Sargramostim, rhu GM-CSF)
1-6	3 µg/kg/day for 4 days
7-12	3 µg/kg/day for 7 days
13-18	6 µg/kg/day for 4 days
19-24	6 µg/kg/day for 7 days

1.7.2 Randomised Controlled Trial

Leukine (Sargramostim, rhu GM-CSF) 3 or 6* µg/kg/day OR placebo administered subcutaneously for 4 or 7* days. *Dose and duration to be decided on basis of dose-finding study.

1.8 Sample size

We plan to recruit a maximum of 24 patients (6 patients x 4 dosing regimens) to the dose-finding study. Sample sizes for the dose finding study were derived pragmatically. Equivalent numbers have provided comprehensive data in similar settings. A sample size of n=6 per group (24 in total) provides 80% power to detect an effect size of 1.8 at a significance level of 0.05 between any 2 groups using the 2 sample t-test.

Based on our previous data (with SD of 13% for primary outcome) our power calculation estimates that for the RCT, a sample size of 17 in each group gives power of 90% to detect a difference (absolute difference in mean phagocytosis of 15%) between the groups at 2 days with a significance level of 0.05 using the 2-sample t-test.[4] To allow for an attrition rate of approximately 10% we would intend to recruit 38 patients (19 per group). This is equivalent to an effect size of 1.15.

1.9 Method of Participant Assignment

Patients entered into the RCT will be individually randomised after informed consent has been obtained and final eligibility confirmed.

1.10 Examination points

A screening blood test for neutrophil dysfunction will take place as a final step for eligibility of entry into both the dose-finding study and the RCT after consent for entry into the study has been received.

Subcutaneous GM-CSF or placebo injection will be administered on day 0 and at the same time on each subsequent study day thereafter up to day 3 or day 6 of both the dose finding study and randomised controlled trial. (The total duration of administration in the RCT will be determined by the results of the dose finding study.)

1.10.1 Dose finding study

During the dose finding study blood will be collected daily for GM-CSF concentration measurement, neutrophil phagocytosis, neutrophil CD88 and monocyte HLA-DR expression, and safety analysis. Urine will be collected daily for GM-CSF concentration measurement. The schedule of study events for the dose finding study is illustrated in the table below.

Table 1. Schedule of events for dose finding study

		Day									
	-1	0	1	2	3	4	5	6	7	8	9
Eligibility assessment	X										
Informed Consent	X										
Neutrophil CD88 Quantification	X										
Study drug		X	X	X	X	X*	X*	X*			
Blood sampling (safety ≤10mls)		X	X	X	X	X	X	X	X	X	X
Blood sampling (phagocytosis ≤ 20mls)		X	X	X	X	X	X	X	X	X	X
Neutrophil CD88 /monocyte HLA-DR expression/ GM-CSF concentration/ neutralising antibodies to GM-CSF (≤5mls)		X	X	X	X	X	X	X	X	X	X
Urine for GM-CSF concentration		X	X	X	X	X	X	X	X	X	X
Adverse events		X	X	X	X	X	X	X	X	X	X

*Whether study drug is given on these days depends on dosing scheduled assigned to patient

1.10.2 Randomised controlled trial

During the RCT blood will be drawn for phagocytosis, neutrophil functional assays, monocyte HLA-DR expression and other tests of inflammation (20mls) on alternate study days up to day 8. Blood will be drawn for safety assessments (<10mls) on a daily basis (these samples will form part of usual clinical care).

The schedule of study events for the RCT is illustrated on the table below.

Table 2. Schedule of study events for RCT

		Day										
	-1	0	1	2	3	4	5	6	7	8	9	10-30
Eligibility assessment	X											
Informed consent	X											
Neutrophil CD88 quantification	X											
Randomisation		X										
Study drug administration		X	X	X	X	X*	X*	X*				

Blood sampling (safety ≤10ml)		X	X	X	X	X	X	X	X	X	X	
Blood sampling (phagocytosis ≤ 20ml)		X		X**		X**		X**		X**		
SOFA score		X	X	X	X	X	X	X	X	X	X	X
ICU status (i.e. whether still in ICU)		X	X	X	X	X	X	X	X	X	X	X
Adverse events		X	X	X	X	X	X	X	X	X	X	
Survival analysis		X	X	X	X	X	X	X	X	X	X	X
ICUAI (by HELICS criteria)		X	X	X	X	X	X	X	X	X	X	X
Neutrophil functional assays, neutralising antibodies to GM-CSF, monocyte HLA-DR expression ≤5mls		X		X**		X**		X**		X**		

*Whether study drug is given on these days will be determined by the dose finding study
** If no trained researcher available to complete analysis blood may be collected the following day

1.11 Primary Outcomes

The primary outcome for the study will be neutrophil phagocytic capacity 2 days after GM-CSF injection.

1.12 Secondary Outcomes

The secondary outcomes of the study will include a) sequential neutrophil phagocytic capacity; b) sequential organ failure assessment score (SOFA); c) length of ICU stay; d) incidence of ICUAIs; e) 30 day mortality; f) other measures of neutrophil function; g) safety; h) duration of mechanical ventilation.

1.13 Assessments

Neutrophil CD88 expression and monocyte HLA-DR expression will be determined by flow cytometry using whole blood.

GM-CSF concentration will be measured in serum and urine by ELISA.

Neutrophil functional assays will be determined following isolation of neutrophils from whole blood by dextran sedimentation and techniques such as percoll gradient separation. Phagocytosis will be determined by techniques such as zymosan ingestion, ROS generation by techniques such as superoxide dismutase inhibitable cytochrome C reduction and neutrophil migration by techniques such as the 'under agarose' method.

1.14 Weekend Blood Sampling

Blood samples for safety analysis will be collected and analysed daily including Saturday and Sunday for all patients. These samples form part of the patient's usual care and will be processed in the hospital lab. Blood samples for neutrophil CD88, neutrophil functional assays and GM-CSF concentration require processing by a trained member of the research group. These samples will only be collected on a Saturday or Sunday when such a person is available or when this represents the day for assessment of the primary outcome (2 days after GM-CSF injection).

2. STUDY TEAM

Chief Investigator (CI)	Prof John Simpson, Professor of Respiratory Medicine, Institute of Cellular Medicine, 4th Floor, William Leech Building, Medical School, Newcastle University, Framlington Place, Newcastle upon Tyne NE2 4HH
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3. BACKGROUND

3.1 Background information

Hospital-acquired infection (HAI) is a major concern for the public, health policy makers and governments.[1] It typically arises in approximately 6% of all hospitalised patients[2, 3] but this figure rises to 20-40% in intensive care units.[4, 5]

A significant effort has been made to address the rates of ICU-acquired infection (ICUAI) in recent years including the introduction of care bundles and improved hand hygiene.[6-8] Despite these measures the overall rate of ICUAI has not significantly improved and continues to be associated with significantly increased morbidity, mortality and cost.[9] In addition ICUAI leads directly to an increased use of antibiotics, in turn promoting the emergence of antibiotic-resistant pathogens which commonly cause ICUAI. Novel approaches to target the problem are required.

Specific pharmacological interventions for the prevention of ICUAI have attracted relatively little attention. This has been due in part to the lack of a unifying model to explain pathophysiology across the range of HAIs. In parallel, redundancy among the many potential molecular mediators of susceptibility to infection has hampered the identification of logical pharmacological targets. This position could be altered significantly if therapies to boost cellular innate immune function were reliably identified.

3.2 Rationale for Study

Dysfunctional Neutrophil Phagocytosis Occurs in Critically Ill Patients

The significant majority of HAIs are caused by bacterial pathogens, though fungi contribute an increasing proportion of cases.[10] In health the circulating blood neutrophil makes by far the greatest contribution to the rapid elimination of bacteria and fungi.[11]

Neutrophil function is significantly impaired in critically ill patients in the ICU.[12] We previously demonstrated that neutrophils from critically ill patients have markedly reduced capacity to phagocytose microbiological particles.[13,14] The problem is compounded by the fact that patients' neutrophils are less capable of generating bactericidal reactive oxygen species (ROS) and have an increased tendency to promote the release of cytotoxic mediators.[13]

Dysfunctional Neutrophil Phagocytosis is Associated with Increased risk of ICUAI

We recently made the important observation that neutrophil phagocytic dysfunction is largely driven by systemic generation of C5a (activated complement factor 5, which acts as an anaphylotoxin).[13] We also showed that CD88 (the receptor for C5a on human neutrophils) is an effective biomarker for neutrophil phagocytic capacity with low CD88 levels correlating directly with neutrophil phagocytic dysfunction.[13,14] We went on to demonstrate that 53% of ICU patients with neutrophil dysfunction developed an ICUAI, as compared with 9% of those patients with good neutrophil function (high neutrophil CD88 expression).[14] These findings are consistent with the increasing recognition that nosocomial infection is associated with a state of 'immune paresis'.

GM-CSF Can Restore Neutrophil Phagocytic Capacity when Applied to Dysfunctional Cells *ex vivo*

Crucially, we have demonstrated that treating patients' dysfunctional neutrophils with granulocyte-macrophage colony stimulating factor (GM-CSF) *in vitro* reproducibly restores phagoctyic function.[13,14] Indeed, in every one of 24 critically ill patients studied, phagocytic function was improved by *ex vivo* application of GM-CSF.[14] The clear association between phagocytic dysfunction and ICUAI, and the restoration of phagocytosis by GM-CSF, suggest that GM-CSF may be able to prevent ICUAI in patients with demonstrable impairment of phagocytosis.

The Intervention has an Acceptable Safety Profile

GM-CSF boosts granulopoiesis and production of monocytes.[15] This has led to GM-CSF being studied and applied in a wide range of clinical settings, with a good safety profile. However remarkably few studies have tested the place of GM-CSF in non-neutropaenic critically ill adult patients, [16-19] and none have specifically studied ICU patients in whom neutrophils have been proven to be dysfunctional prior to administration.

GM-CSF is an FDA-approved drug licensed for use in the treatment of patients following chemotherapy for acute myeloid leukaemia and post bone marrow transplant. The doses selected for use in this trial are within the recommended doses for use within these indications.

3.3 Local Confirmation of Neutrophil CD88 Expression Flow Cytometry Assay

Our work illustrating the correlation of neutrophil surface CD88 expression with phagocytic capacity was carried out in a laboratory in Edinburgh. This study will be completed in Newcastle upon Tyne using the flow cytometry facilities at Newcastle Medical School.

In order to ensure that we are selecting the correct, at risk, patient group we will carry out a pre-study local confirmation of the neutrophil CD88 assay in our current laboratory by comparing CD88 expression with phagocytic capacity in a group of 30 critically ill patients who would meet the criteria for entry into the clinical trial.

Consent will be gained from all patients (or from a personal or professional legal representative where the patient is deemed to be lacking in capacity) prior to entry into this section of the study. Where consent is obtained for participation a single 20ml blood sample will be taken from the patient. Where possible the blood sample will be collected from an in-dwelling line in order to minimise discomfort to the patient. If no in-dwelling lines are in situ the blood will be collected from a peripheral vein. 18mls of the blood collected will be used to isolate neutrophils for assessment of phagocytic capacity and 2 mls will be used to perform a CD88 flow cytometry assay. The results will be plotted against each other to determine the level of CD88 expression correlating with impaired neutrophil phagocytic capacity (<50% neutrophils ingesting 2 or more zymosan particles).

All samples collected will be labelled with a unique anonymous study number. Only the age and sex of the patients will be recorded. No other data will be collected on these patients. Their involvement in the study will cease following the collection of the single 20 ml blood sample. There will be no alterations in their usual clinical care. Patients entered in other non-pharmacological intervention studies may be included.

4. AIMS AND OBJECTIVES

4.1 Primary aim

- This is a non-commercial study. The study's primary aim, as a proof of concept application, is to test the hypothesis that GM-CSF targeted to critically ill patients, with known neutrophil dysfunction, restores effective neutrophil phagocytosis.

4.2 Secondary aims and objectives

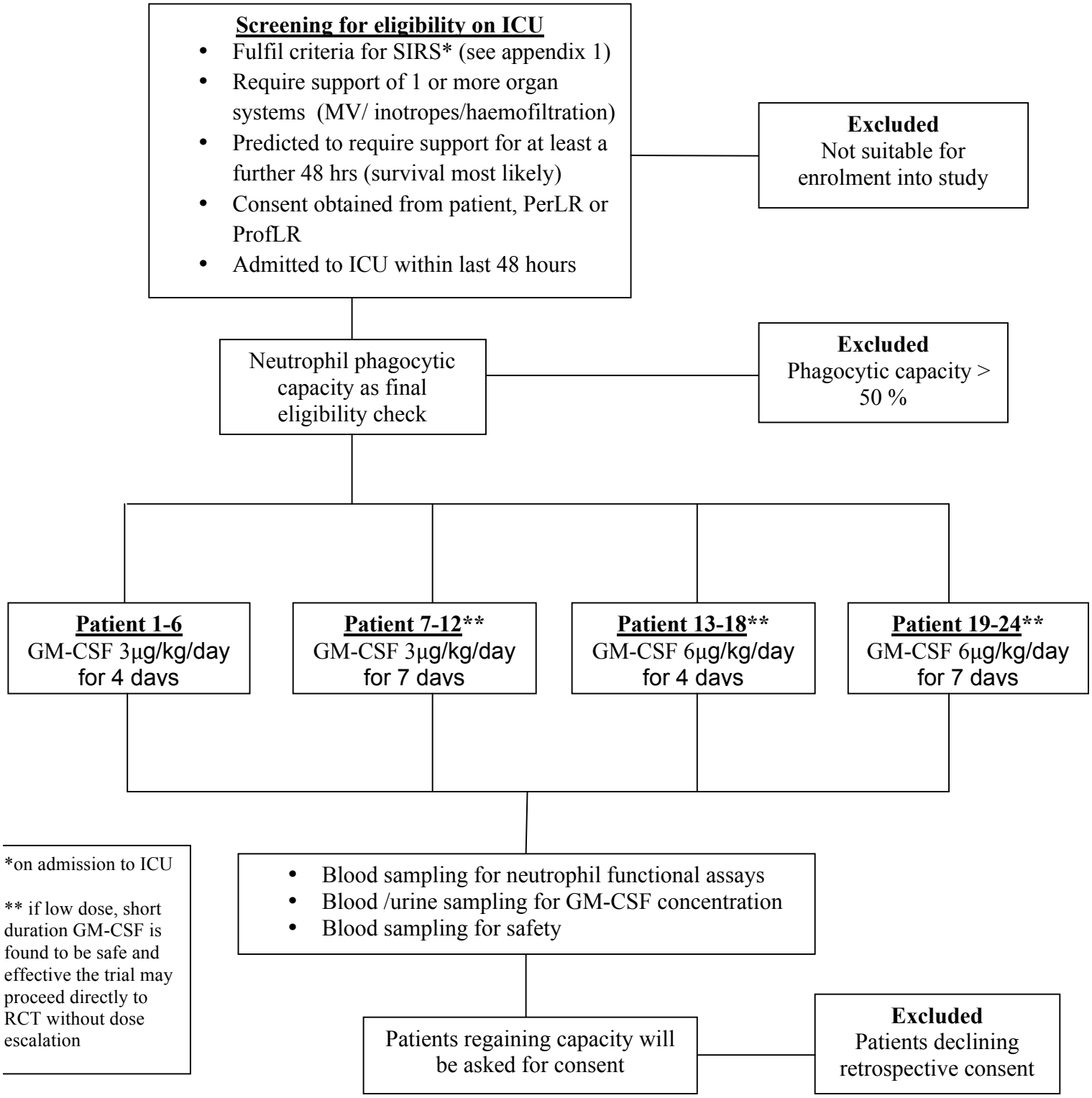
- To establish the optimum dose/duration of GM-CSF needed to restore dysfunctional neutrophil phagocytosis in critically ill patients and to establish the sustainability of any effect.
- To assess the effect of GM-CSF on other elements of neutrophil function (which may include but not be limited to superoxide generation, transmigration and apoptosis).
- To assess whether GM-CSF has beneficial effects in terms of length of ICU stay, duration of mechanical ventilation and 30-day mortality.
- To assess whether GM-CSF administration is associated with a lower incidence of ICUAI in those patients who are randomised to receive it.

5. STUDY DESIGN

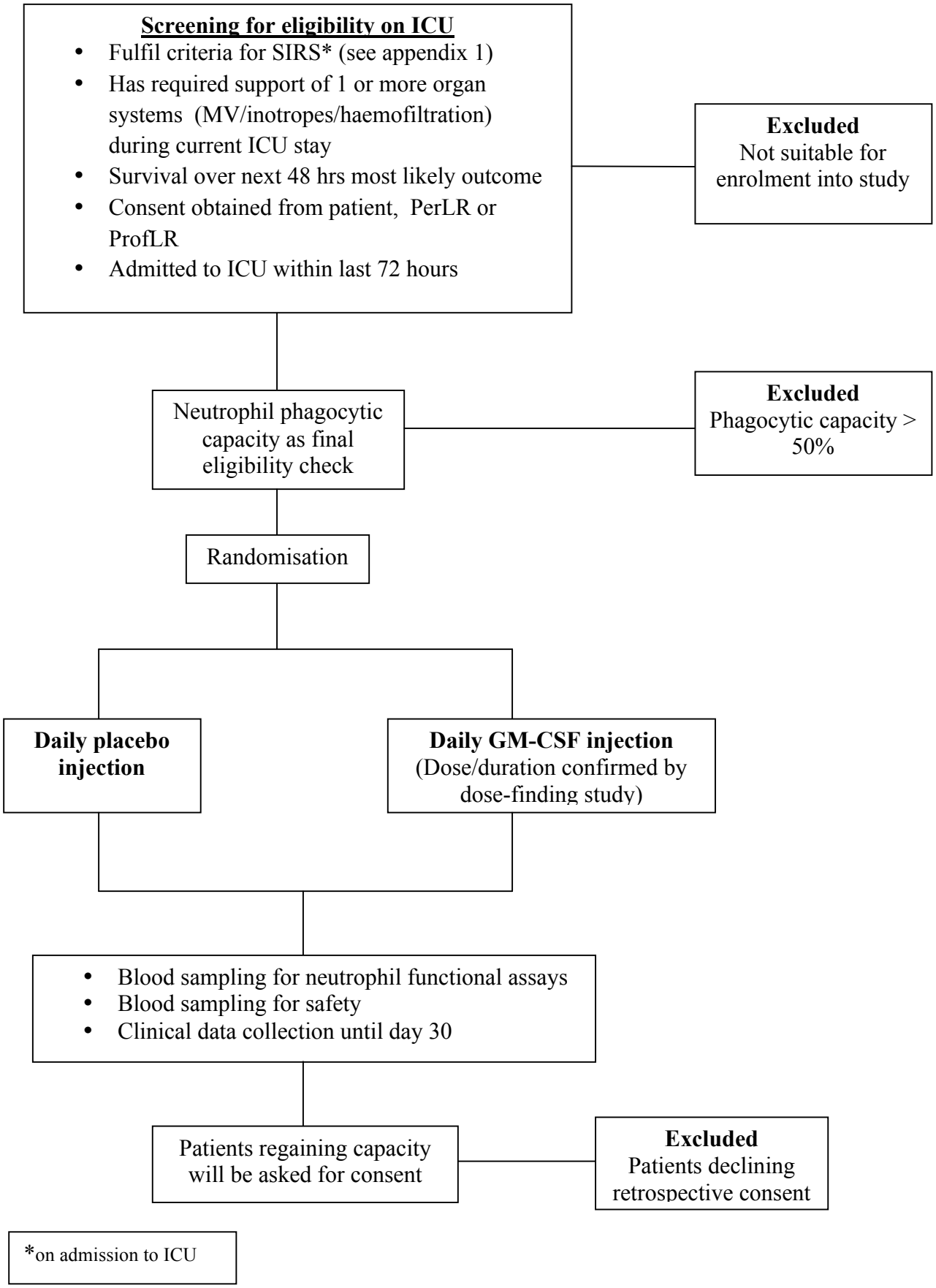
5.1 Design of Study

The study involves two distinct components. The first component is an open labelled dose finding study. The second component is a prospective, randomised parallel group, double-blind, placebo controlled trial.

5.2.1 Schematic Diagram for Dose-finding Study



5.2.2 Schematic diagram for RCT



5.3 Study sites

Patients will be screened in 4 ICUs in England, the Royal Victoria Infirmary (RVI) and the Freeman Hospital (FRH), Newcastle upon Tyne, the Queen Elizabeth Hospital (QEH), Gateshead and Sunderland Royal Hospital (SRH). Two sites (RVI and FRH) are large teaching hospitals, the other sites (QEH & SRH) are district general hospitals. Involvement of all four sites will increase the relevance of study data to the general ICU population. The RVI and FRH are located within 2 miles of each other and are part of the same trust and therefore Dr Baudouin will act as PI for both sites.

5.4 Study Patients

5.4.1 Inclusion criteria

Patients fulfilling **all** of the following criteria will be eligible for entry into the study.

- Fulfil criteria for systemic inflammatory response syndrome on admission to ICU (see appendix 1)
- Has required support of one or more organ systems (invasive ventilation, inotropes or haemofiltration) during current ICU stay
- Survival over next 48 hours deemed most likely outcome by responsible ICU clinician
- Admitted to ICU within last 72 hours
- Neutrophil phagocytic capacity <50%

5.4.2 Exclusion criteria

The presence of **any** of the following criteria will exclude entry to the study

- Absence/refusal of informed consent
- Current prescription of a colony stimulating factor
- Any history of allergy/adverse reaction to GM-CSF
- Total white cell count >$30x10^9$/litre at time of screening
- Haemoglobin < 7.5g/dl at the time of screening
- Age < 18 years
- Pregnancy or lactation
- Known in-born errors of neutrophil metabolism
- Known haematological malignancy and/or known to have >10% peripheral blood blast cells
- Known aplastic anaemia or pancytopaenia
- Platelet count <$50x10^9$/litre
- Chemotherapy or radiotherapy within the last 24 hours
- Solid organ or bone marrow transplantation
- Use of maintenance immunosuppressive drugs other than maintenance corticosteroids (allowed up to 10mg prednisolone/day or equivalent)
- Known HIV infection
- Active connective tissue disease (e.g. rheumatoid disease, systemic lupus erythematosus) requiring active pharmacological treatment.
- ST-segment elevation myocardial infarction, acute pericarditis (by ECG criteria) or pulmonary embolism (radiographically confirmed) in previous week
- Involvement in any study involving an investigational medicinal product in the previous 30 days

5.5 Duration of Study

For the dose finding study a maximum of 24 patients, completing a full dosing regimen, will be recruited over approximately 6 months. During the randomised controlled trial 38 patients will be recruited over approximately 10 months. Following randomisation patients will participate actively in the dose finding study and randomised controlled trial for up to 9 days, including daily contact with the trial team. Retrospective consent will be sought from patients once they have recovered capacity. Clinical follow up data for patients in the RCT will be collected at 30 days but no contact will be made with the patient at this time point.

5.6 Trial Interventions

5.6.1 Dose-finding study

Patients in the dose finding study will receive a daily dose of subcutaneous Leukine (Sargramostim, rhu GM-CSF) as detailed below for 4 or 7 days.

Patient Enrolment Number	Dose of Leukine (Sargramostim, rhu GM-CSF)
1-6	3 µg/kg/day for 4 days
7-12	3 µg/kg/day for 7 days
13-18	6 µg/kg/day for 4 days
19-24	6 µg/kg/day for 7 days

5.6.1.1 Dose-escalation scheme

6 patients will be recruited to each dosing schedule. Screening will take place on each unit on a daily basis and those patients who meet the eligibility criteria and consent to participation in the study (or whose PerLR/ProfLR consent) will be recruited consecutively. We expect recruitment to be proportional across the sites. Within each dose cohort the first patient recruited will be treated and observed for 3 days after the initial injection of Leukine prior to the entry of the next patient. Following this patients 2,3,4,5 and 6 may be entered. The safety and tolerability data collected during the treatment and observation period for all six patients in the cohort (up to day 9 following initial injection of leukine) will be evaluated prior to dose-escalation. The data will be sent to the Data Monitoring and Safety Committee (DMSC) 5-10 days after the last dose of Leukine (Sargramostim, rhu GM-CSF) is administered to patient 6 within the cohort. If 2 of the 6 patients in a cohort experience a dose-limiting toxicity (see section 5.6.1.2) during the treatment period dose-escalation will not take place.The decision to dose-escalate or not will be made by the DMSC. Minutes will be recorded at the dose-escalation decision meetings and circulated for final approval before being placed on file. The same procedure will be followed for each subsequent dosing schedule. The minimum time interval between dosing schedules (ie dosing of the last patient in one cohort and the first patient in the next will be 7 days).

If low dose, short duration (3ug/kg/day for 4 days) GM-CSF is demonstrated to be effective (ie we observe a phagocytosis rate on day 2 that is protective against infection (≥50% in all patients) and if there is no toxicity observed, then we will proceed directly to RCT at that dose. Higher doses would then seem unlikely to further reduce infection risk significantly, while carrying an increased risk of toxicity.

5.6.1.2 Dose-limiting toxicity

A dose-limiting toxicity will be considered as any serious adverse event which is judged to be probably or definitely related to the administration of Leukine (Sargramostim, rhu GM-CSF).

5.6.1.3 Selection of optimum dose for the randomised controlled trial

The dose selected for use in the randomised controlled trial will be that which is shown to be effective ie produces a demonstrable increase in phagocytosis and in which <2 of the 6 patients in the cohort experience a dose-limiting toxicity.

5.6.2 Randomised controlled trial

Patients enrolled in the RCT will be randomised to receive a daily subcutaneous injection of Leukine (Sargramostim, rhu GM-CSF) 3 or 6 µg/kg/day OR placebo for 4 or 7 days. The final dose and duration of study drug or placebo will be decided following the outcome of the dose-finding study.

5.7 Outcome Measures

5.7.1 Primary Outcome Measure

The primary outcome measure is neutrophil phagocytic capacity 2 days after administration of GM-CSF/placebo (as measured by the percentage of neutrophils ingesting ≥ 2 zymosan particles *ex vivo*).

5.7.2 Secondary Outcome Measures

There are a number of secondary outcomes based on biological, clinical and safety measures.

5.7.2.1 Biological measures

- Sequential neutrophil phagocytic capacity on alternate study days (to determine sustainability of any observed effects in the primary endpoint)
- Neutrophil phagocytic capacity measured, for example, as 'area under the curve' over the study period
- Other assessments of neutrophil function which may include, but are not limited to, ROS generation, migratory capacity and apoptotic rate
- Monocyte HLA-DR expression on alternate study days
- Serum measures of inflammatory response which may include, but not be limited to, cytokine levels

5.7.2.2 Clinical Measures

In addition to baseline clinical and demographic measurements the following data will be recorded:

- Sequential organ failure assessment (SOFA) score
- Length of stay in ICU
- The incidence of ICUAIs (as defined by Hospitals in Europe Link for Infection Control Surveillance (HELICS) criteria – see appendix 4)
- All cause mortality 30 days post randomisation
- Number of days of mechanical ventilation

5.7.2.3 Safety measures

Safety measurement will be made in relation to effect on:

- Full blood count – including haemoglobin level and platelet count
- White cell count including neutrophil, monocyte, eosinophil and lymphocyte counts
- U&Es and LFTs
- Development of neutralising antibodies to GM-CSF

Serious adverse events (SAEs) and occurrence of suspected unexpected serious adverse reactions (SUSARs) as defined in section 11 will be recorded.

6. INVESTIGATIONAL MEDICINAL PRODUCT (including Product information leaflet/ SmPC equivalent & Product Monograph – appendix 2 & 3)

6.1 General Description

GM-CSF is one of a group of growth factors termed colony stimulating factors. It acts by stimulating the differentiation and division of progenitor cells in the granulocyte-monocyte pathway.[15] This includes neutrophils, the key cell in bacterial and fungal pathogen defence and the focus of study in this trial.

Recombinant human GM-CSF is clinically available in 2 forms

i) a glycosylated compound derived from a yeast based system
ii) a non-glycosylated compound prepared in an *E. coli* based system.

The effects of the two compounds on granulopoiesis are broadly comparable[20] but the yeast-derived product has a superior safety profile and has therefore been selected for use in this trial.[21]

6.2 License and Indications

The investigational medicinal product to be used in this study is the recombinant human GM-CSF, Leukine (Sargramostim, rhu GM-CSF). It is a glycosylated compound derived from a yeast based system. It is owned by Genzyme (a subsidiary of Sanofi-Aventis) and is an FDA-approved drug for the treatment of patients post chemotherapy or bone marrow transplant where it aids the recovery of the myeloid cells and reduces the incidence of serious infection..[22]

6.3 Manufacture and Supply

Sargramostim is manufactured in the U.S., to GMP requirements, by Bayer, under the trade name Leukine. It is produced by recombinant DNA technology in a *Saccharomyces cerevisiae*-based system. It is a glycoprotein of 127 amino acids characterized by three primary molecular species having molecular masses of 19,500, 16,800 and 15,500 daltons. The amino acid sequence of Leukine differs from natural human GM-CSF by a substitution of leucine at position 23.

The drug will be imported by Mawdsley Brooks and Co., Quest 22, Quest Park, Silk Road, Off Wheatley Hall Road, Doncaster, DN2 4LT. They will provide certification by a Qualified Person (QP) that the manufacturing site works in compliance with GMP. The will also provide a copy of the importers manufacturing authorization as required by the MHRA.

6.4 Preparation and Storage

Sargramostim is presented as a liquid or a lyophilized white powder for reconstitution with sterile water for injection and is suitable for both subcutaneous and intravenous injection. The lyophilized powder form will be used in this trial. It should be stored in a refrigerator between 2-8°C. It will be reconstituted with sterile water for injections from a licensed UK market source. When reconstituted with sterile water the lyophilized form must be used within 6 hours. If reconstituted with bacteriostatic water the lyophilized form may be stored for up to 20 days The reconstituted product appears as a clear, colourless liquid. Sub-cutaneous administration appears to result in a more gradual accumulation and decline in serum concentrations and has been selected as the route of administration for use in this trial.[23, 24]

6.5 Clinical Pharmacology

Leukine has species-specific biological effects. *In vitro* studies using human bone marrow cells have shown a dose-dependent response in terms of progenitor cell proliferation, [25] and an increase in chemotaxis and anti-fungal and anti-parasitic activities of neutrophils and monocytes.[26] Concentrations ranging from 1-100ng/ml have been studied.
Pharmacological and toxicological studies carried out on monkeys showed no major visceral organ pathology following either single dose or daily dosing up to one month by either intravenous or subcutaneous preparations using doses up to 200 micrograms/kg/day. As with *in vitro* human studies dose dependent responses were seen in terms of leucocyte proliferation, with counts recovering to baseline within 1 -2 weeks.[27]
Pharmacokinetic studies have shown that when Leukine was administered s/c to healthy volunteers GM-CSF was detectable in the blood at 15 minutes, peak concentrations occurred between 1 and 3 hours and Leukine remained detectable for up to 6 hours post injection.[27]

6.6 Clinical Experience

Acute myeloid leukaemia (AML)

Leukine is used in the treatment of older (55-70 years) neutropaenic patients with AML post chemotherapy where it shortens neutrophil recovery and reduces the incidence of, and death rate from, serious infections.[28]

Bone Marrow Transplantation

Studies of GM-CSF in the treatment of patients post autologous and allogenic bone marrow transplant show significant improvements in the rate of myeloid engraftment duration of hospital stay and frequency of infections. A shorter duration of antibiotic therapy was also seen in those patients receiving Leukine post bone marrow transplant. It is used widely in this context in the U.S.[27, 29-31]

Sepsis

The largest area of literature in the use of GM-CSF in sepsis relates to the care of neutropaenic premature babies where it enhances neutrophil recovery and possibly reduces nosocomial infection rates but has no impact on survival.[32-35]
Few studies have looked at the use of GM-CSF in the treatment of non-neutropaenic septic adults, however some recently published studies have shown effects in terms of various parameters of sepsis-related immune dysfunction in addition to non-significant benefits in terms of length of stay, duration of mechanical ventilation and FiO_2:PaO_2.[16-19] No published trials have studied the use of GM-CSF in patients with demonstrated neutrophil dysfunction.

Other Indications

Immunomodulatory properties of GM-CSF have shown varying degrees of promise in prostate cancer, Crohn's disease, human immunodeficiency virus (HIV) infection, childhood neuroblastoma and vaccination strategies.[36-41] Promising topical use of GM-CSF has been described for burns, cervical intraepithelial neoplasia and pulmonary alveolar proteinosis.[42-45] However, with the possible exception of prostate cancer, so far there is insufficient evidence to recommend widespread application of GM-CSF in these settings.

6.7 Contraindications

Leukine is contraindicated in the following patients (see Product Information Leaflet /SmPC equivalent, and Product monograph appendix 2 & 3)

i) any patient with known hypersensitivity to GM-CSF or yeast derived products

ii) any patient with >10% blast cells in the bone marrow or peripheral blood

iii) any patient who has received chemotherapy or radiotherapy in the previous 24 hours

Such patients have been excluded from entry into the study to minimise risk of harm.

6.8 Side Effects / Toxicity Profile.

6.8.1 IMP clinical trial literature relevant to the study population

The dose of GM-CSF used in research and in clinical practice tends to be in the range of 3-10 μg/kg/day. In reported studies in critically ill non-neutropaenic adults, doses have ranged between 3μg/kg/day for 3 days through to 4 μg/kg/day for 8 days.[16-19] The emerging consensus is that GM-CSF has a dose-dependent adverse event profile, with low toxicity rates if doses less than 10 μg/kg/day are used.[50] Published data does not suggest detrimental effects in terms of organ function.

A study by Meisel et all looking at the effects of GM-CSF on sepsis induced monocyte function included 38 critically ill patients treated with doses of 4 mcg/kg/day GM-CSF for 8 days. They reported that no adverse effects were seen, even in a sub-group of patients whose doses were increased to 8mcg/kg/day following a lack of improvement in monocyte HLA-DR expression.[19] In relation to organ dysfunction and disease severity; duration of mechanical ventilation, length of ICU stay and length of intra-hospital stay were all shorter in patients receiving GM-CSF. Despite patients in the GM-CSF group having higher

vasopressor support at baseline noradrenaline requirements were lower in this group after the intervention interval. In addition there were no differences seen in the need for renal replacement therapy between the two groups. Baseline mean APACHE II and SOFA scores were higher in the control group, however, scores fell in both groups over the treatment period with greater improvements seen in the GM-CSF group.[19]

Similarly a study by Presneill et al of 10 critically ill patients with respiratory compromise on ICU treated with 3mcgm/kg/day of GM-CSF for 5 days reported only 1 adverse event relating to an elderly gentleman with sepsis-related renal dysfunction who developed transient oliguria following administration of the 3rd dose. The drug was discontinued after the 4th dose and his renal function recovered.[18] The authors found no other evidence of worsening organ dysfunction in the GM-CSF group during the study and reported an improvement in oxygenation in the treated patients.[18]

A third study of adult non-neutropaenic patients with abdominal sepsis recorded 3 adverse events in 28 patients treated with 3mcg/kg/day of *E. coli*-derived GM-CSF for 4 days. This included 1 episode of thrombocytopaenia, 1 rash and 1 patient treated for nausea.[16]

6.8.2 IMP clinical trial literature related to common indications (including Product Information Sheet/SmPC equivalent & Product Monograph appendix 2 & 3)

Over all Leukine is generally well tolerated. Recognised associated adverse events include fever, chills, bone pain, myalgia, headache and erythema at the injection site. Large uncontrolled studies involving more than 200 patients recorded these adverse effects as being mild to moderate in nature and reversed by the use of simple analgesics such as paracetamol.[28]

In placebo controlled studies involving more than 150 bone marrow transplant patients similar rates of side effects were experienced by those receiving Leukine as those receiving placebo. In addition studies involving patients with AML found the only significant difference in the rate of adverse events was an increase in skin-associated events in those receiving Leukine. [28]
Genzyme includes several warnings in their product literature for potentially serious adverse events reported with the use of the product. They advise caution in the following situations: [28]

Pre-existing renal/hepatic disease - A rise in serum creatinine, bilirubin and hepatic enzymes has been seen in some patients with pre-existing hepatic or renal disease enrolled in uncontrolled trials. Ceasing treatment with Leukine or reducing the dose has resulted in values returning to pre-treatment levels. In controlled trials, however, the incidences of renal and hepatic dysfunction were comparable between Leukine and placebo treated patients. Product literature suggests monitoring of renal and hepatic function at least every other week during Leukine administration. All patients enrolled in the study will have daily U&E and LFT monitoring.

Fluid retention - Oedema, capillary leak and pleural/pericardial effusions have been observed in patients treated with GM-CSF and it is thought that it may aggravate fluid retention in those with pre-existing disease. In 156 patients enrolled in placebo controlled studies incidences of such symptoms were as follows (Leukine vs placebo) peripheral oedema 11% vs 7%, pleural effusion 1% vs 0%, pericardial effusion 4% vs 1%. Capillary leak syndrome was not observed in these studies but based on other uncontrolled studies and

reports from users of marketed Leukine, the incidence is estimated to be less than 1%. Again it has been observed to be reversible with interruption or reduction in dose.

Cardiovascular disease – There are some reports of occasional transient supra-ventricular tachycardia, particularly in patients with pre-existing cardiac arrhythmias. Caution in such patients is advised. All patients on ICU will be on a cardiac monitor for the duration of the study as part of their usual care.

Respiratory complications have been noted among patients treated with intravenous infusions of Leukine. Dyspnoea and sequestration of granulocytes have occasionally been observed. The lack of such reports in recent studies on critically ill patients with s/c GM-CSF may be related to the method of administration with more gradual rises in peak concentration. Subcutaneous administration of GM-CSF will be used throughout this study. The majority of patients in this study are likely to be invasively ventilated and all will have saturation and regular blood gas monitoring throughout.

6.8.3 IMP clinical trial literature related to other Indications

In an RCT of GM-CSF using 6 μg/kg/day s/c for 56 days in Crohn's disease, overall adverse events were similar in treatment and placebo arms. Bone pain and injection site reaction were more common (but injection site haematoma less common) with GM-CSF treatment.[37] Allergic reactions have been described.[46] Serious adverse events such as pericarditis and thrombosis have only been described with doses in excess of 20 μg/kg/day.[24]

6.8.4 First Dose Syndrome

It is recognised that a syndrome characterised by flushing, tachycardia, dyspnoea, hypotension and respiratory distress can be seen following the first administration of Leukine in a cycle. The features have resolved with symptomatic treatment and are not seen to recur with further administration.[28]

6.8.5 Antibody formation

The development of neutralising antibodies to GM-CSF is known to occur particularly in the context of prolonged administration. Two large studies found rates of 2.3% (n=214) and 1.3% (n=75). The clinical relevance of these antibodies is not known at present. Patients in both components of the trial will be screened on alternate days for the development of neutralising antibodies.[28]

6.9 Placebo

The placebo study drug will be normal saline for subcutaneous injection. All normal saline used in the trial will be from a licensed UK market source. The drug/placebo will be prepared in a blinded fashion and the final appearance of the drug and syringes will be identical in terms of volume, labelling and appearance of the solution.

6.10 Labelling of IMP

The labelling of the IMP will be performed by Mawdsley Brooks and Co. in compliance with the applicable regulatory requirements including annex 13.

6.11 Prescription of IMP

Trial delegation logs will specify the names of physicians authorised to prescribe the IMP for this study

6.12 Disposal and Accountability

The relevant hospital pharmacy will keep an accurate record of the dates and amounts of study drug dispensed. Where a dose of study drug is not administered it will be returned to the relevant hospital pharmacy. At the end of the study unallocated and unused study drug will be destroyed with permission from the Sponsors and in accordance with the site pharmacy procedure for destruction of an IMP. A record of the destruction will be maintained.

6.13 Study drug provision at the end of the study

The study drug is being investigated for use as a short term intervention during critical illness. The study drug will therefore not be provided beyond the clinical trial. Usual clinical care will resume on completion of the study drug.

7. STUDY PROCEDURES

7.1 Screening Procedure

Patients will be prospectively screened on ICU, on the basis of the inclusion/exclusion criteria as specified in this protocol (section 5.4.1-5.4.2, page 16). The screening procedure will be identical for both the dose finding study and RCT. Screening may be performed by any qualified individual(s) designated by the local PI and listed on the delegation log as having responsibility for this aspect of the study.

Each site PI will be responsible for maintaining a screening log. Entries may be made by any qualified individual(s) designated by the local PI. If a screened patient is not recruited the reason for not being enrolled must be recorded on the screening log. The study includes some time-dependent issues. The pattern of neutrophil function/dysfunction in critically ill patients appears to be established within the first 48 hours after admission to ICU. The previous work defining the independent predictive risk of cellular biomarkers in this clinical scenario used this time point for entry into studies.[13, 14] To allow for recruitment of all such at risk patients, including those admitted at weekends and those in whom the need for organ support is delayed, we have placed a limit on screening to occur within the first 72 hours of admission to ICU.

7.2 Informed Consent Procedure

The study will be conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. Eligible patients may only be included in the trial after obtaining written informed consent. Informed consent must be obtained prior to conducting any trial specific procedures and the process for obtaining informed consent must be documented in the patient's medical records.

Patient information sheets and informed consent forms approved by the Research Ethics Committee (REC) will be provided to each study site. The incapacitating nature of the condition precludes obtaining prospective informed consent from all, or nearly all, participants. In this situation informed consent will be sought from a Personal Legal Representative (PerLR), or from a Professional Legal Representative (ProfLR) should no PerLR be available. The PI is responsible for ensuring that informed consent for study participation is given by each patient or a legal personal or professional representative.

The time dependent factors discussed above in relation to screening (section 7.1, page 21) will be explained to the patient, PerLR or ProfLR. They will be given up to 24 hours to make a decision regarding consent but will be asked to provide a decision within 6 hours if they feel able.

In cases where the patient or PerLR may not adequately understand verbal explanations or written information given in English the relevant hospital's translation service will be called upon to provide all necessary oral and/or written translation.

An appropriately trained doctor or nurse may take consent. If no approved form of consent is obtained a patient cannot enter/be randomised into the study.

7.2.1 Personal Legal Representative Consent

If the patient is unable to give informed consent, consent may be sought from a patient's PerLR who may be a relative, partner, carer or close friend. The PerLR will be informed about the trial by the responsible clinician or a member of the research team and they will be provided with a copy of the Covering Statement for the PerLR with an attached Participant Information Sheet (PIS) and asked to give an opinion as to whether the patient would object to taking part in such medical research. If the PerLR decides that the patient would have no objection to participating in the study then they will be asked to sign 2 copies of the PerLR Consent Form, which will then be countersigned by the person taking consent. A copy of the signed consent form and the PIS will be placed in the patient's medical records, whilst the originals will be retained by the PerLR and by the PI in the Investigator Site File (ISF).A copy will also be retained to be given to the patient once they have regained capacity.

7.2.2 Professional Legal Representative Consent

If the patient is unable to give informed consent and no PerLR is available a doctor who is not directly connected with the conduct of the trial (i.e. not named in section 2 page 11) may act as a ProfLR. The doctor will be informed about the trial by the responsible clinician or a member of the research team and given a copy of the (PIS) If the doctor decides that the patient is suitable for entry into the trial then they will be asked to sign 2 copies of the ProfLR Consent Form. A copy of the signed consent form and the PIS will be placed in the patient's medical records, whilst the originals will be retained by the ProfLR and by the PI in the ISF.A copy will also be retained to be given to the patient once they have regained capacity.

7.2.3 Retrospective Patient Consent

Patients will be informed of their participation in the study by the responsible clinician or a member of the research team once they regain capacity to understand the details of the study. The decision as to whether capacity has returned will rest with the medical team caring for the patient. The responsible clinician or a member of the research team with delegated responsibility (as per delegation log) will discuss the study with the patient and he/she will be given a copy of the Patient Information Sheet to keep. The patient will be asked for consent to participate in the study and to sign two copies of the retrospective consent form. A copy of the signed retrospective consent form will be placed in the patient's medical records whilst the originals will be retained by the patient and by the PI in the ISF. If the patient refuses consent no further data will be collected about or from the patient. The patient will then be asked to consent to whether or not data collected up to that point in the study can be entered into the analysis.

7.3 Withdrawal of Consent

Patients may withdraw or be withdrawn (by PerLR or ProfLR) from the study at any time without prejudice. Data recorded up to the point of withdrawal will be included in the study analysis, unless consent to use data already collected has also been withdrawn.

7.4 Patient registration and randomisation procedure

7.4.1 Dose finding study

After informed consent has been received, patients with confirmed low neutrophil phagocytic capacity will commence treatment with daily s/c GM-CSF. The dose and duration of treatment will be determined by the sequence of entry into the study as outlined in section 5.6.1, page 17.

7.4.2 Randomised controlled trial

After informed consent has been received, patients with confirmed low phagocytic capacity will be randomised to receive daily s/c GM-CSF or placebo. Randomisation will be in a 1:1 ratio, with stratification by site, using a web-based randomisation service in NCTU. The randomised allocation schedule will be generated by a statistician with no other involvement in the study to ensure independence and concealment of allocation. Permuted blocks of variable length will be used to reduce the risk of breach of concealment of allocation. A treatment number will be generated for each participant that links to the corresponding allocated study drug/placebo. The treatment number will be clearly documented by the investigator on the trial prescription to ensure the study pharmacist dispenses the correct study medication.

Contact details for randomisation: http://apps.ncl.ac.uk/random/ (available 24hrs)

7.5 Administration of Investigational Medicinal Product/Placebo Injections

As previously described the yeast-derived form of rhu GM-CSF, Leukine (Sargramostim) will be used in both the dose-finding study and RCT (section 6, page 18). It will be provided as a lyophilised powder which will be reconstituted in sterile water for injection from a licensed UK market source. Once reconstituted, the drug will be administered within 6 hours. The placebo drug will be normal saline from a licensed UK market source.

The first dose of the study drug (Leukine (Sargramostim, rhu GM-CSF) or placebo) will be given, on the morning of entry into the study, after final eligibility has been confirmed by phagocytic capacity assessment. Subsequent doses will be given at the same time each day thereafter. If for any reason a dose is not administered at the intended time, it may be administered subsequently but not more than 6 hours after the intended time of administration. Administration of GM-CSF/placebo injections will be carried out by any trained clinical member of the research or usual care team according to the product administration guidelines contained in the Product Information Leaflet (SmPC equivalent/ product monograph Appendix 2&3).

The dose of GM-CSF for the RCT will be decided on the basis of the dose finding study as described in section 5.6.

7.6 Study Drug Termination Criteria

The study drug (Leukine (Sargramostim, rhu GM-CSF).) will be continued until one of the following conditions is met (whichever comes first):

- maximum treatment period (to be defined in the dose finding study for RCT)
- study drug-related SAR
- discharge from a critical care environment
- death
- discontinuation of active medical treatment
- the patient, PerLR or ProfLR requests withdrawal from the study
- decision by the attending clinician that the study drug should be discontinued on safety grounds.

In the following situations consideration will be given to either discontinuing the study drug or reducing the dose by half to minimise the risk of complications

- WCC > 50,000 cells/mm3
- Platelet count > 500,000 cells/mm3

7.7 Blood sampling

Up to 20ml of blood will be drawn from existing lines (≤ 20mls for phagocytosis assays, ≤10mls for safety analysis, ≤5mls for flow cytometry/ELISA assays). Where possible this will be from arterial lines. Where arterial lines are inaccessible, venous lines may be used (and this information recorded). In the event that all indwelling vascular access devices are removed prior to completion of the study blood will be drawn by venesection no more than once per day in addition to the patient's usual care.

7.8 Clinical Management of Patients in the Study

Administration of either GM-CSF or a placebo injection will be outside of usual clinical care for all patients entered into the study.

Blood drawn for assessment of phagocytosis, other elements of neutrophil function, monocyte HLA-DR expression and cytokine multiplex will be outside of usual clinical care for patients entering into the study.
Blood drawn for safety assessment including full blood count (FBC), urea and electrolytes (U&E) and liver function tests (LFTs) would be included in usual patient care for all patients on ICU even if they were not included in the study.

All other aspects of usual patient care will be delivered to patients prior to, during and after completion of the study.

7.9 Study Procedures for Unblinding

Each participant will be randomly allocated to receive either study drug or placebo. As a placebo controlled, double-blind trial, patients, clinicians and the PIs will be blinded to each patient's allocation. All trial drugs, whether GM-CSF or placebo, will be packaged identically at the point of administration and identified only by a unique trial identifier. Either PI may request emergency unblinding on grounds of safety. Emergency unblinding will be performed by the use of sealed code-break envelopes which will be held within the hospital pharmacy at the relevant study site. The envelopes will be available for access 24 hours a day.

Where the PI at the relevant site is not available the CI should be contacted to authorise unblinding. In an emergency situation where no contact can be made with the CI or PI (e.g. out of hours) unblinding should proceed and the CI should be informed at the first available opportunity.

To arrange unblinding the duty or on-call pharmacist should be contacted via the relevant hospital switchboard.

8. STUDY ASSESSMENTS

8.1 Clinical Assessments

In addition to the outcome measurements described in Section 5.7.2.2 (page 18) the following baseline details will be recorded on the appropriate Case Report Forms (CRFs): age; date of (and reason for) admission to hospital; date of (and reason for) admission to ICU; Acute Physiology and Chronic Health Evaluation) APACHE II score at admission; medications; co-morbidities; sequential organ failure assessment (SOFA) score; BP; PaO_2:FiO_2 ratio (FiO2/Sats if no arterial line); full blood count; urea and electrolytes; liver function tests; and blood glucose. These data will also be recorded on each study day up to day 10 and then at days 20 and 30 when the study data will be completed for each patient.

8.1.1 Dose finding study

The schedule of study events for the dose finding study is illustrated on the table below.

Table 1. Schedule of events for dose-finding study

		Day									
	-1	0	1	2	3	4	5	6	7	8	9
Eligibility assessment	X										
Informed Consent	X										
Neutrophil CD88 Quantification	X										
Study drug		X	X	X	X	X*	X*	X*			
Blood sampling (safety ≤10mls)		X	X	X	X	X	X	X	X	X	X
Blood sampling (phagocytosis ≤20mls)		X	X	X	X	X	X	X	X	X	X
Neutrophil CD88 /monocyte HLA-DR expression/ GM-CSF concentration/neutralising antibodies (5≤mls)		X	X	X	X	X	X	X	X	X	X
Urine for GM-CSF concentration		X	X	X	X	X	X	X	X	X	X
Adverse events		X	X	X	X	X	X	X	X	X	X

*Whether study drug is given on these days depends on dosing scheduled assigned to patient

8.1.2 Randomised controlled trial

During the RCT blood will be drawn for phagocytosis, neutrophil functional assays, monocyte HLA-DR expression and other tests of inflammation (20mls) on alternate study days up to day 8. Blood will be drawn for safety assessments (<10mls) on a daily basis (these samples will form part of usual clinical care).

The schedule of study events for the RCT is illustrated on the table below.

Table 2. Schedule of study events for RCT

		Day										
	-1	0	1	2	3	4	5	6	7	8	9	10-30
Eligibility assessment	X											
Informed consent	X											
Neutrophil CD88 quantification	X											
Randomisation		X										
Study drug administration		X	X	X	X	X*	X*	X*				
Blood sampling (safety ≤10ml)		X	X	X	X	X	X	X	X	X	X	
Blood sampling (phagocytosis ≤ 20ml)		X		X**		X**		X**		X**		
SOFA score		X	X	X	X	X	X	X	X	X	X	X

ICU status (i.e. whether still in ICU)		X	X	X	X	X	X	X	X	X	X	X
Adverse events		X	X	X	X	X	X	X	X	X	X	
Survival analysis		X	X	X	X	X	X	X	X	X	X	X
ICUAI (by HELICS criteria)		X	X	X	X	X	X	X	X	X	X	X
Neutrophil functional assays, neutralising antibodies to GM-CSF, monocyte HLA-DR expression ≤5mls		X		X**		X**		X**		X**		

*Whether study drug is given on these days will be determined by the dose finding study
** If no trained researcher available to complete analysis blood may be collected the following day

8.2 Blood

Blood samples will be collected as described in section 7.9, page 23. Blood samples for neutrophil CD88, neutrophil functional assays and GM-CSF concentration require processing by a trained member of the research group. These samples will only be collected on a Saturday or Sunday when such a person is available or when this represents the day for assessment of the primary outcome (2 days after GM-CSF injection).

8.2.1 Preparation of Neutrophils from Whole Blood

Neutrophils will be separated from whole blood by recognised techniques such as dextran sedimentation and percoll gradient technique.

8.2.2 Neutrophil Phagocytosis Assay

Neutrophil phagocytosis will be determined by recognised techniques such as zymosan ingestion. The percentage of neutrophils ingesting 2 or more zymosan particles will be recorded by microscopic analysis of 4 fields of neutrophils each containing at least 100 cells.

8.2.3 Neutrophil CD88 Expression

Neutrophil CD88 expression will be determined in whole blood, using flow cytometry.

8.2.4 Monocyte HLA-DR Expression

Monocyte HLA-DR expression will be determined in whole blood, using flow cytometry.

8.2.5 GM-CSF Concentration

GM-CSF concentration in serum will be measured by recognised techniques such as ELISA.

8.2.6 GM-CSF Neutralising Antibodies

GM-CSF neutralising antibodies will be assayed by recognised techniques such as ELISA.

8.2.7 Neutrophil Reactive Oxygen Species Production

Neutrophil reactive oxygen species production will be measured by recognised techniques such as superoxide dismutase inhibitable cytochrome C reduction.

8.2.8 Neutrophil migration

Neutrophil migration will be assessed by recognised techniques such as the under agarose method.

8.2.9 Other Analyses

Serum will be frozen at temperatures of at least -20°C for later analyses such as assessment of cytokines and other assays of inflammation/infection as appropriate.

8.3 Urine

Urine will be collected from an indwelling urinary catheter in a universal container.

8.3.1 Urine GM-CSF Concentration

Urine GM-CSF concentration will be measured by recognised techniques such as ELISA.

9. COLLECTION AND STORAGE OF DATA

9.1 Recording of data

All data for an individual patient will be collected by each PI or their delegated nominees and recorded in the case report form (CRF) for the study. Patient identification on the CRF will be through a unique study identifier number. A record linking the patient's name to the unique study identifier number will be held only in a locked drawer at the study site, and is the responsibility of the PI. As such, patients cannot be identified from CRFs. Copies of CRFs will be made – one will be kept in the ISF and the other will be retained by the Newcastle Clinical Trials Unit (NCTU). The NCTU will continually audit completeness and quality of data recording in CRFs and will correspond regularly with site PIs (or their delegated assistants) with the aim of capturing any missing data where possible, and ensuring continuous high quality of data.

Data will be collected and recorded on the CRF by site research teams from the time the patient is considered for entry into the study through to the completion of outcome data. In the event that a patient is transferred to another hospital, the site research team will liaise with the receiving hospital to ensure complete data collection.

Clinical information will not be released without the written permission of the participant, except as necessary for monitoring and auditing by the Sponsor, its designee, Regulatory Authorities, the Data Monitoring and Safety Committee (DMSC) or the REC. Secure anonymised electronic data may however be released to the Study Statistician for analysis. The PI and study site staff involved with this study may not disclose or use for any purpose

other than performance of the study, any data, record, or other unpublished, confidential information disclosed to those individuals for the purpose of the study. Prior written agreement from the Sponsor or its designee must be obtained for the disclosure of any said confidential information to other parties.

9.2 Data Management

Data received at NCTU will be processed as per the CTU SOPs, including entering the data into a secure central database. Responsibility for maintenance of the database will rest with the study manager.

9.3 End of Study

Up to a maximum of 24 patients, completing one of the four dosing regimens, will be recruited to the dose finding study. Dose escalation may stop prior to completion of all four dosing regimens if a satisfactory dose response is seen at one of the lower doses or if unacceptable side effects are observed. Recruitment will cease after 38 patients have entered the RCT. For regulatory and ethical reporting purposes, end of study is defined as when follow-up outcome data (30 days) is collected for the last patient.

The study will stop sooner than this if:

- mandated by the relevant REC
- mandated by the MHRA
- mandated by the Sponsor (for example on the advice of the DMSC)
- funding was withdrawn.

10. STORAGE OF SAMPLES

Samples of serum and plasma will be labelled with the unique study identifier number and stored frozen. As described above the patient's identity cannot be determined from the unique study identifier number alone. Samples will be processed and stored in Newcastle, where they will be kept in a locked freezer in the CI's research facility. Samples will be stored for up to 15 years after completion of the study. Consent will be obtained for sample storage.

Flow cytometry samples will be discarded after use. Cytospins will be performed, and the glass slides (labelled using only the unique study identifier number) will be stored in the CI's lab for up to 15 years after completion of the study.

11. PHARMACOVIGILANCE

Timely, accurate and complete reporting and analysis of safety information from clinical trials is crucial for the protection of patients and is mandated by regulatory agencies.

11.1 Definitions

Term	Definition
Adverse Event (AE)	Any untoward medical occurrence in a patient or clinical trial subject administered a medicinal product and which does not necessarily have a causal relationship with this treatment.
Adverse Reaction (AR)	All untoward and unintended responses to an investigational medicinal product related to any dose administered
Unexpected Adverse Reaction (UAR)	An adverse reaction the nature or severity of which is not consistent with the applicable product information (e.g. investigator's brochure for an unauthorised investigational product or SmPC for an authorised product).
Serious Adverse Event (SAE) **Serious Adverse Reaction (SAR)** **Suspected Unexpected Serious Adverse Reaction (SUSAR)**	Respectively, any adverse event, adverse reaction or unexpected adverse reaction that: a)results in death ; b)is life-threatening (i.e. the subject was at risk of death at the time of the event; it does not refer to an event which might have caused death if it were more severe); c)requires hospitalisation or prolongation of existing hospitalisation; d)results in persistent or significant disability or incapacity; e)is a congenital anomaly or birth defect; f)is any other important medical event(s) that carries a real, not hypothetical, risk of one of the above outcomes

11.2 Assessment of Causality

Each AE should be clinically assessed for causality based on the information available, i.e. the relationship of the AE to the study drug should be established. The assignment of the causality should be made by the principal investigator responsible for the care of the participant using the definitions in the table below. All adverse events judged as having a reasonable suspected causal relationship to the study drug (i.e. definitely, probably or possibly related) are considered to be adverse reactions. If any doubt about the causality exists, the local Principal Investigator should consult the Chief Investigator. In the case of discrepant views on causality between the Principal Investigator and others, all parties will discuss the case and will refer as necessary to the DMSC. In the event that no agreement is reached the main REC and other bodies will be informed of both points of view.

Relationship	Description
Unrelated	There is no evidence of any causal relationship. The clinical event has an incompatible time relationship to the study drug administration, and could be explained by underlying disease, or other drugs or chemicals.
Unlikely	There is little evidence to suggest there is a causal relationship (e.g. the event did not occur within a reasonable time after study drug administration). There is another reasonable explanation for the event (e.g. the participant's clinical condition).
Possible	There is some evidence to suggest a causal relationship (e.g. the event occurs within a reasonable time after the study drug administration). However the influence of other factors may have contributed to the event (e.g. the participant's clinical condition).
Probable	There is evidence to suggest a causal relationship, including a reasonable time relationship with the study drug administration, and the influence of other factors is unlikely.
Definitely	There is clear evidence to suggest a causal relationship and other possible contributing factors can be ruled out.
Not assessable	There is insufficient or incomplete evidence to make a clinical judgement of the casual relationship.

11.3 Adverse Event Reporting Period

The AE reporting period for this trial begins upon enrolment into the trial and ends 28 days following administration of the study drug. All AEs assessed by the PI as possibly related to the study drug and all SAEs that occur during this time will be followed until they are resolved or are clearly determined to be due to a patient's stable or chronic condition or intercurrent illness(es).

11.4 Adverse Event Reporting Requirements

AEs should be reported and documented on the relevant pages of the CRF, in accordance with the procedures outlined below. The PI, or a delegated nominee, at each site will evaluate all AEs for expectedness in addition to causality.

11.5 Expected Adverse Events

Most adverse events that occur in this study, whether they are serious or not will be expected, treatment-related toxicities due to the study drugs, or non-treatment related due to the underlying critical illness. A full list of expected undesirable side effects of Leukine, Sargramostim, are outlined above (sections 6.8.1.- 6.8.5, page 21) and in the Product Information Leaflet. (SmPC equivalent/Product monograph Appendix 2&3)

11.6 Reporting AEs

Because this trial is recruiting a population that is, by definition, already in a life-threatening situation, it is expected that many of the participants will experience AEs. Events that are expected in this population (i.e. events in keeping with the patient's underlying medical condition) should not be reported as AEs. An adverse reaction (AR) is an AE which is related to the administration of the study drug. If any AEs are related to the study drug (i.e. are ARs) they must be reported on the AE form within the CRF. Adverse events occurring within 1 hour of administration of the study drug should be considered related. It is the responsibility of the PI to record all relevant information in the CRF.

The following are ARs which are expected and must be reported on the AE form within the CRF:

- skin reaction at the site of injection
- fever up to 38° C
- elevated WCC > 50,000 cells/mm^3
- bone pain
- myalgia
- antibody formation
- 'first dose syndrome' (as described in section 6.8.5, page 22)

An unexpected adverse reaction (UAR) is an AE which is related to the administration of the study drug and that is unexpected, in that it has not been previously reported in the current Product Information Leaflet. All UARs must be reported on the AE form within the CRF.

Unexpected adverse events will be included as part of the safety analysis for the trial and do not need to be reported separately to the CTU.

11.7 Reporting Serious Adverse Events

Because this study is recruiting a population that is already in a life-threatening situation, it is expected that many of the participants will experience SAEs. Events that are expected in this population (i.e. events in keeping with the patient's underlying medical condition) and that are collected as outcomes of the trial, including death and organ failure, should not be reported as SAEs. Other SAEs must be reported. A serious adverse reaction (SAR) is an SAE which is related to the administration of the study drug. These must be reported to the CTU.

Suspected unexpected serious adverse reactions (SUSARs) are SAEs that are considered to be caused by the study drug and are unexpected i.e. their nature and severity is not consistent with the SmPC.

If an SAR occurs, reporting will follow the regulatory requirements as appropriate and all SUSARs will be the subject of expedited reporting. SAEs will be evaluated by the PI for causality (i.e. their relationship to the study drug) and expectedness. Once the PI becomes aware that an SAR has occurred in a study patient, they must complete the SAE form in the CRF and report the information to the NCTU within 24 hours. The SAE form must be completed as thoroughly as possible with all available details of the event, signed by the PI or designee. If the PI does not have all information regarding an SAE, they will not wait for this additional information before notifying NCTU. The form can be updated when the additional information is received. Follow up information should include whether the event

has resolved, if and how it was treated and whether the patient continues in the study or has been withdrawn from treatment.

The SAE form should be transmitted by fax to the NCTU on 0191 2228901.

The NCTU is responsible for reporting SUSARs (i.e. SAEs that are considered to be related and unexpected) to the Sponsors, the REC, and the MHRA within 15 days of becoming aware of the event using the NRES Reporting of SAE Form. In the event of a fatal or life threatening SUSAR reporting to the relevant regulatory authorities should take place within 7 days.

The Co-ordinator of the main REC should acknowledge receipt of related, unexpected safety report within 30 days.

11.8 Reporting SAEs to Data Monitoring and Safety Committee

SAEs will be reported to the DMSC within the same timelines as for Regulatory reporting. A copy of each report will be sent to the DMSC.

11.9 Pregnancy

It is not known whether Sargramostim can cause foetal harm if administered to pregnant women.[28] All pregnant women will be excluded from entry into the study. A pregnancy test will be performed on all women of child-bearing potential prior to final confirmation of eligibility and those with a positive pregnancy test will be excluded. In the unlikely event, that it is subsequently confirmed, that a participant was pregnant at the time of the study despite a negative pregnancy test, then follow up will continue until completion of the pregnancy.

12. STATISTICAL CONSIDERATIONS

12.1 Sample Size

Sample sizes for the dose finding study were derived pragmatically. Equivalent numbers have provided comprehensive data in similar settings. A sample size of n=6 per group (24 in total) provides 80% power to detect an effect size of 1.8 at a significance level of 0.05 between any 2 groups using the 2 sample t-test.

The sample size for the RCT was calculated based on our previous data studying the effects of GM-CSF on *ex-vivo* phagocytosis in a cohort of critically ill patients.[14] The mean rate of 'neutrophils ingesting ≥ 2 zymosan particles' in ICU patients was 39% (standard deviation (SD) 13%) and was associated with a high rate of subsequent ICUAI. Therefore we believe an effect size incorporating an absolute increase of at least 15% (i.e. from 39% to 54% mean phagocytosis) is clinically important. To place this in context, in our hands *ex-vivo* treatment of patients' neutrophils with GM-CSF resulted in 68% of neutrophils ingesting ≥ 2 zymosan particles i.e. an absolute increase of 29%. We therefore have considerable confidence in the ability to achieve this effect size. On the basis of these data our power calculation estimates that a sample size of 17 in each group gives power of 90% to detect such a difference

(absolute difference in mean phagocytosis of 15% between the groups at 2 days) with a significance level of 0.05 using the 2-sample t-test. To allow for an attrition rate of approximately 10% we would intend to recruit 38 patients (19 per group). This is equivalent to an effect size of 1.15.

This sample size would provide 90% power to detect an effect size of 1.15 at a significance level of 0.05 (e.g. with respect to a comparison of change from baseline between the 2 groups, where an estimate of the change in SD is not available from the literature).

12.2 Data Analysis

12.2.1 Dose finding study

The dose finding study, incorporating a small number of patients is designed to assess safety, feasibility and tolerability. It is not designed to enable, or powered for, comparative hypothesis testing and therefore only basic descriptive statistics (including frequencies, means, medians, standard deviations, interquartile ranges and proportions/rates as appropriate) together with associated confidence intervals will be presented to summarise the outcome data. These summaries will be presented by dose level, with safety data being reviewed prior to dose escalation.

12.2.2 RCT

As this is a phase 2 clinical study, with relatively small numbers of participants, we intend to report descriptive statistics (such as rate estimates for any dichotomous or categorical variables and means, medians, standard deviations and inter-quartile ranges for continuous measures) with appropriate confidence intervals in order to inform future study design. Summaries of demographic data will also be reported.

We will also explore comparative analysis of the primary endpoint (neutrophil phagocytic capacity at 2 days after administration of GM-CSF/placebo) between the treatment groups although, due to the sample size, this will be exploratory in nature rather than definitive. Analysis will be performed using Analysis of Covariance (ANCOVA) or similar methods in order to allow for the effects of covariates, including any stratification factors. We shall also examine change from baseline and other time periods for this outcome variable in a similar manner.

Several outcomes will be evaluated to determine whether treatment with GM-CSF shows efficacy for surrogate biological and clinical outcomes. Secondary endpoints will be examined using broadly similar methods. However, dichotomous variables will be analysed in an exploratory fashion using techniques such as logistic regression. Although the incidence of ICUAIs and 30-day mortality will be documented, these important clinical endpoints are not included as major outcome variables as this proof of concept study is not adequately powered to assess these (they are expected to be the focus of trials leading on directly from this work).

Data analysis will be on an intention to treat basis, although other exploratory analyses (such as on a per-protocol basis) may also be considered. Data with missing observations (other than due to mortality) will be examined to determine both the extent of and reason for such omissions. Multiple imputation techniques will be used as appropriate to allow for this in the analysis.

13. REGULATIONS, ETHICS AND GOVERNANCE

13.1 Sponsorship

The study will be sponsored by Newcastle upon Tyne Hospitals NHS Foundation Trust.

13.2 Regulatory and Ethical Approvals

The study will be conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. A favourable ethical opinion from a Research Ethics Committee and R&D approval via the NIHR Coordinated System for gaining NHS Permissions is a prerequisite prior to commencement of the study. MHRA approval is also a prerequisite prior to commencement of the study. Local R&D approval will be secured before recruitment may commence at each site. The NCTU will require a written copy of local approval documentation, before sites recruit patients into the study. The study will be registered with the UK National Institute for Health Research (NIHR) Clinical Research Portfolio. In order that the study remains on the NIHR Portfolio and receives the appropriate level of support through the relevant Local Research Network, accrual data on patient recruitment will be forwarded to the UK Clinical Research Network (UKCRN) Co-ordinating Centre on a monthly basis by the NCTU. The study will be registered on the European Clinical Trials Database (EudraCT) and other public databases.

13.3 Ethical Considerations

The vulnerability of this study group is fully appreciated and every effort will be undertaken to protect patients' safety and well-being.

13.4 Protocol Compliance

The investigators will conduct the study in compliance with the protocol given favourable opinion by the REC and the MHRA. Amendments to the protocol will require favourable opinions from the relevant ethics committee, R&D and MHRA prior to implementation, except when modification is needed to eliminate an immediate hazard(s) to patients. The NCTU in collaboration with the Sponsor will submit all protocol modifications to the REC and MHRA for review in accordance with the governing regulations. Protocol compliance will be monitored by the trial manager who will ensure that the trial protocol is adhered to and that necessary paperwork (CRF's, patient consent) are being completed appropriately. Any deviations from the protocol will be fully documented in source documentation and in the CRF.

13.5 Patient Confidentiality

In order to maintain confidentiality, all CRFs, stored samples and study reports will identify patients by the assigned unique study identifier number only. The only link between the patient's identity and the unique study identifier number will be held at the relevant study site, in a locked drawer with restricted access.

13.6 Good Clinical Practice (GCP)

The study will be carried out in accordance with the principles of the International Conference on Harmonisation Good Clinical Practice (ICH-GCP) guidelines (www.ich.org). The CI, site PIs, clinical research associate and study nurse must have completed GCP training and have up to date certification before recruitment begins.

13.6 Study Monitoring

13.6.1 Direct Access to Data

The agreement with each PI will include permission for study-related monitoring, audits, ethics committee review and regulatory inspections, by providing direct access to source data and study-related documentation. Consent from patients/legal representatives for direct access to data will also be obtained. Patient confidentiality will be maintained and will not be made publicly available to the extent permitted by the applicable laws and regulations.

13.6.2 Monitoring Arrangements

The NCTU will be responsible for study monitoring. On-site monitoring visits will be conducted in accordance with the study's monitoring plan. Before the study starts at either participating site, an initiation visit will take place to ensure that essential documents and trial supplies are in place and that site staff are fully aware of the study protocol and SOPs. On-site monitoring visits during the study will check the completeness of patient records, the accuracy of entries on CRFs, the adherence to the protocol, SOPs and GCP, and the progress of patient recruitment.

Site PIs should ensure that access to all study related documents including source documents (to confirm their consistency with CRF entries) are available during monitoring visits. The extent of source data verification will be documented in the monitoring plan.

13.7 Indemnity

The participating NHS Trusts have liability for clinical negligence that harms individuals toward whom they have a duty of care. Indemnity in respect of negligent harm arising from study management is provided via NHS schemes by the Newcastle Upon Tyne NHS Foundation Trust in its role as sponsor. Indemnity in respect of negligent harm arising from study conduct is provided by NHS schemes, via the participating NHS Trusts, covering NHS-employed staff and medical academic staff with honorary NHS contracts, who are conducting the trial. Indemnity in respect of negligent harm arising from study design or protocol authorship is provided by NHS schemes, for those protocol authors whose substantive contract of employment lies with the NHS, and via University insurance schemes for protocol authors who have their substantive contract with a University. This is a non-commercial study and there are no arrangements for non-negligent compensation.

13.8 Finance

The study is funded by the Medical Research Council through the Developmental Clinical Studies scheme (study funding reference number G1100233).

13.9 Record Retention

Each site PI will be provided with an ISF by the NCTU and will maintain all study records according to GCP and the applicable regulatory requirements. The study master file (SMF) will be held by the NCTU and the essential documents that make up the file will be listed in a SOP. On completion of the trial the SMF and study data will be archived by the NCTU according to the applicable regulatory requirements and for up to 15 years. The ISFs and all study record files held by the PIs will be transferred to the custody of the CI. Following confirmation from the Sponsor the NCTU will notify the CI when they are no longer required to maintain the files. If the CI withdraws from the responsibility of keeping the study records, custody must be transferred to a person willing to accept responsibility and this must be conveyed in writing to NCTU.

14. STUDY COMMITTEES

14.1 Study Management Arrangements

The CI will have overall responsibility for the conduct of the study. The NCTU will be the Study Co-ordinating Centre. The NCTU will provide study management and coordination, data management and monitoring. The study manager will be responsible on a day-to-day basis for overseeing and co-ordinating the work of the multi-disciplinary study team.

14.2 Project Management Group (PMG)

The PMG will be considered quorate if it contains at least

- the CI,
- the Project Manager,
- one of the site PIs,

though all members of the study group will be welcome to attend. Members of the DMSC will also be invited.

The PMG will meet at approximately 3 monthly intervals. Additional meetings can be called if risks, important changes or unexpected costs are identified. Management responsibility between meetings rests with the CI, assisted by the Project Manager.
The PMG will report to the DMSC, and to public/patient groups (for example CritPal).

14.3 Data Monitoring and Safety Committee

A DMSC will be appointed comprising at least two independent clinicians with experience in undertaking clinical studies and caring for patients with haematological and critical illness, and a statistician, all of whom are independent of the study. The DMSC will convene 1 week after completion of each dosing schedule within the dose-finding study and thereafter approximately every 3 months. The DMSC's responsibility is to safeguard the interests of the study participants, in particular with regard to safety, and to assist and advise the PMG so as

to protect the validity and credibility of the study. The DMSC will receive reports allowing them to monitor recruitment, adverse events and outcome data. Where the DMSC members consider that cessation of the study should be considered or mandated, the Sponsor will be promptly informed.

14.4 User Involvement

The study will be registered with the INVOLVE open-access database (http://www.involve.org.uk). The PMG will also report to the Chairman of the Patient Liaison Committee of the Intensive Care Society (CritPaL). CritPaL will be invited to comment on elements of the study including Participant Information Sheets and dissemination of results.

15. PROPOSED STUDY MILESTONES

Formal milestones are in place for this study and have been agreed with the funders (Medical Research Council). Timely recruitment to the study requires entry of approximately 1 patient per week between the 4 ICUs. An interim analysis at 2 months will assess whether recruitment is proceeding to target. The PMG will assess whether recruitment is satisfactory.

16. DISSEMINATION

The data generated from the trial will be the property of the Chief Investigator and the Co-investigators. Publication will be the responsibility of the Chief Investigator. The findings will be presented at national and international meetings and we aim to publish the findings in high quality peer-reviewed open access journals.

A lay person's summary of the principal findings of the results will be sent to all patients involved in the study at their request. In addition a lay person's summary will be sent to CritPaL. A report of the study findings will be sent to the INVOLVE registry.

17. LIST OF APPENDICES

Appendix 1 – Criteria for the diagnosis of SIRS

Appendix 2 – Product Information Leaflet for Leukine (Sargramostim, rhu GM-CSF).

Appendix 3 – Product Monograph

Appendix 4 – HELICS Criteria for the diagnosis of ICUAI : Hospitals in Europe Link for Infection Control through Surveillance: Surveillance of Nosocomial Infections in Intensive Care Units: Protocol version 6.1, September 2004. Case definitions of ICU-acquired infections, pages 7-11.

18. REFERENCES

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APPENDIX 1

Criteria for the diagnosis of SIRS

Two of the following must be present

- Temperature < 36 or >38 C
- Heart rate > 90 bpm
- Tachypnoea with respiratory rate > 20 breaths per minute or $PaCO_2$ < 4.3kPa
- White blood cell count $<4x10^9$/L (<4000/mm³) or $>12x10^9$/L (>12,000/mm³), or 10% bands

American College of Chest Physicians /Society of Critical Care Medicine Consensus Conference 1992.