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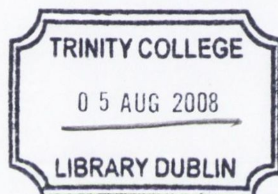
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Cytokine gene expression, protein expression and genetic variability in the human response to infection

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For my parents and for my wife.

We meet people who have crossed deserts, floated on icecaps and cut their way through jungles – and yet in whose souls we would search in vain for evidence of what they have witnessed. Dressed in pink and blue pyjamas, satisfied within the confines of his own bedroom, Xavier de Maistre was gently nudging us to try, before taking off for distant hemispheres, to notice what we have already seen.

Alain de Botton – The Art of Travel

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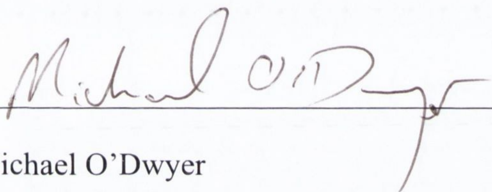

Dr Michael O'Dwyer

Summary

In this thesis I explore the hypothesis that a pro-inflammatory cytokine immune response is a beneficial and protective host mechanism in the setting of the human response to infection. The various regulatory pathways involved in the control of this response are subsequently elucidated.

Utilising the technique of quantitative real-time polymerase chain reaction, gene expression, at a messenger RNA level (mRNA), was assessed for a number of genes intimately involved in inflammation. Protein levels were also assayed using an enzyme linked immunosorbant assay technique and candidate genes were assessed for polymorphisms utilising either an amplifluor or taqman technique. Three patient groups were recruited which consisted of; 62 intensive care unit (ICU) patients with severe sepsis or septic shock (ICU group); 10 patients, from the general hospital wards, with a microbiologically confirmed gram negative bacteraemia and no organ failure or signs of an impending septic crisis (bacteraemia group); 13 healthy staff members (control group).

Tumor necrosis factor alpha (TNFα) mRNA levels were elevated in the ICU group in comparison with the control group but were further elevated in the bacteraemia group. Similarly, interferon gamma (IFNγ) mRNA was elevated in the bacteraemia group in comparison to the ICU group whilst interleukin 10 (IL-10) mRNA was greatest in the ICU group. In late sepsis there was an inverse relationship between both IFNγ and TNFα gene expression and severity of illness and mortality.

Although there was a weak correlation between IFNγ and IL-12 mRNA levels, IL-12 mRNA did not distinguish between the three groups nor did its levels predict mortality. Consequently, we felt it was reasonable to assess the expression of alternative genes linked to T helper cell development. We found that both IL-23 and

IL-27 mRNA levels distinguished between the three groups. The ICU group demonstrated the lowest levels of IL-23 mRNA and the greatest levels of IL-27 mRNA. In addition, there was a strong correlation between IL-23 and TNFα in late sepsis. IL-18 mRNA levels were not predictive of response to a septic insult. Interestingly, mRNA levels of the inhibitor of kappa B-like gene (IκBL), a putative member of the IκB family, also distinguished between the three groups with levels highest in the ICU group and intermediate in the bacteraemia group.

Protein levels were generally undetectable, with the exception of IL-6 and asymmetric dimethyl arginine (ADMA), both of which were directly related to the extent of organ compromise.

We also observed a relationship between promoter polymorphisms in the TNFα gene and the dimethylarginine dimethylaminohydrolase II gene and TNFα mRNA levels and ADMA protein levels respectively.

Overall, these data suggest that, in the setting of infection, a vigorous proinflammatory response is appropriate and may prove to allow patients to tolerate a bacteraemic insult with impunity. The regulation of this response is likely to be multifaceted and involve multiple inflammatory cell pathways, with genetic variants possibly playing a role in the variations observed in gene expression.

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Related publications

Chapters 4 and 5 contain data previously published in *Shock* and this article is reproduced in full in the appendix (A9).

The occurrence of severe sepsis and septic shock are related to distinct patterns of cytokine gene expression. *Shock* 2006 Dec;26 (6) :544-50.

Chapter 7 contains data previously published in *Critical Care* and this article is reproduced in full in the appendix (A10).

Septic shock is correlated with asymmetrical dimethyl arginine levels, which may be influenced by a polymorphism in the dimethylarginine dimethylaminohydralase 2 gene: a prospective observational study. *Critical Care* 2006; 10(5):R139.

Chapter 5 contains data previously published in *Intensive Care Medicine* and this article is reproduced in full in the appendix (A11).

The human response to infection is associated with distinct patterns of interleukin 23 and interleukin 27 gene expression. *Intensive Care Medicine* 2008 Apr; 34(4):683-691.

Chapter 8 contains data accepted for publication in *The International Journal of Immunogenetics*.

Characterisation of tumor necrosis factor-alpha genetic variants influencing gene expression in patients with severe sepsis.

List of abbreviations

ADMA – asymmetrical dimethyl arginine
ALT – alanine transaminase
APC – antigen presenting cell
APTT – activated partial thromboplastin time
Bp – base pair
cDNA – complementary DNA
CLP – cecal ligation and puncture
CNS – central nervous system
Ct – crossing threshold
DC – dendritic cell
DDAH – dimethylarginine dimethylaminohydrolase
dNTP – deoxynucleotide triphosphate
E - efficency
EDTA – ethylenediaminetetraacetic acid
ELISA – enzyme linked immunosorbant assay
FiO_2 – inspired oxygen concentration
IBD – inflammatory bowel disease
ICU - intensive care unit
IFNγ – interferon gamma
Iκβ – inhibitory of kappa beta
IκBL – Inhibitor of kappa B like
IKK - Iκβ kinase
IL – interleukin
INR - international normalised ratio
IQR – interquartile range
kDa – kilodaltons
kg – kilogram
kPa - kilopascal
l – litre
LPS - lipopolysaccaride
MCS – multiple cloning site
MHC – major histocompatibility complex
ml – millilitre
mmHg – millimetres of mercury
MMLV – moloney murine leukaemia virus
μmol – micromole
mmol – millimole
MODS – multiple organ dysfunction score
NFκB – nuclear factor kappa beta
NO – nitric oxide
NOS – nitric oxide synthase
PAI-1 – plasminogen activator inhibitor –1
$PaCO_2$ – alveolar partial pressure of carbon dioxide
PaO_2 – alveolar partial pressure of oxygen
PBMC – peripheral blood mononuclear cell
PMN – polymorphonuclear leukocyte
QRT-PCR – quantitative real time polymerase chain reaction

RTI – respiratory tract infection
SAPS2 – simplified acute physiology score 2
SIRS – systemic inflammatory response syndrome
SNP – single nucleotide polymorphism
SOCS – suppressor of cytokine signalling
SOFA - sequential organ failure assessment score
TBE – Tris borate EDTA
TCR – T cell receptor
Th1 – T helper cell type 1
Th2 – T helper cell type 2
TNFα – tumor necrosis factor-alpha
UTI – urinary tract infection
WCC – white blood cell count

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Chapter 1 - Introduction

1.1 Overview

Acute infection in humans has a scope of presentation that ranges in severity from a mild self-limiting illness to an overwhelming insult with resultant multiple organ failure frequently culminating in death. At the less severe end of the spectrum is an illness that may be amenable to home therapy with oral antibiotics and the patient may not even present to a hospital. However, at the other end of the spectrum is a devastating disease process, representing a large proportion of an intensive care unit's (ICU) workload and budget. It is estimated that the ICU treatment of sepsis utilises 60% of an ICU budget whilst afflicting 25% of it's patients (van Gestel et al., 2004). The mortality attributable to sepsis in an ICU setting varies between 20% to greater than 50% depending on an individual centers expertise and case mix (Angus et al., 2001).

Critically, in the majority of cases, it does not appear that the pathogenicity of the invading micro organism determines the host outcome. Identical strains of bacteria may cause disease of varying severity in different immunocompetent hosts. Presently, it is unclear as to the attributes that lead a host to become susceptible to developing severe sepsis in response to an infection and it is equally unclear what factors lead to the subsequent demise of the host once they have developed severe sepsis.

By studying groups of patients that respond with varying severity to comparable infectious insults we hope to be able to identify the molecular pathways involved in the susceptibility to severe sepsis and in individual patient response to infection.

1.2 Definitions

Traditionally, sepsis has been a vague diagnosis, incorporating a plethora of signs and symptoms. Prior to standardisation of the terminology used, it was a disorder frequently diagnosed by a clinician on the basis of a very non-specific clinical picture. Whilst this approach has proved useful in the identification of many other heterogenous conditions, a more rigid approach was called for with the recent increase in experimental and epidemiological work in sepsis. Standardisation of the definitions involved has allowed investigators confidence in the interpretation of results from sepsis trials conducted in different centers and facilitates direct comparisons.

A consensus conference, convened in 1991, produced the common definitions which are used to characterise sepsis today and are outlined below (1992).

The systemic inflammatory response syndrome (SIRS) follows a wide range of insults and includes more than one of the following clinical manifestations:

1) body temperature >38°C or <36°C

2) heart rate of >90 beats/minute

3) tachypnea, manifested by a respiratory rate >20 breaths/minute or hyperventilation, indicated by a partial pressure of CO_2 ($PaCO_2$) < 32mmHg

4) White Blood Cell count (WCC) >12000 cells/mm^3, < 4000/mm^3, or the presence of >10% immature neutrophils.

These should relate to an acute change in an otherwise normal patient. As such, they should occur in the absence of mitigating circumstances that would otherwise explain such changes. Such circumstances would include conditions such as chemotherapy-induced neutropenia or leukopenia, tachycardia secondary to pain or tachypnea secondary to a primary lung disorders such as a pneumothorax.

Many physiological insults lead to SIRS including pancreatitis, burn injury, ischaemia, multitrauma and tissue injury, haemorrhagic shock, and immune-mediated organ injury.

It is only when SIRS occurs as a result of a confirmed infectious process can it is termed sepsis.

Sepsis, however, is not a homogenous disorder but rather can present over a range of clinical severity. The consensus conference attempted to identify some clearly defined points along this continuum. These include severe sepsis and septic shock.

Severe sepsis was defined as sepsis in combination with organ dysfunction, hypoperfusion or hypotension. Hypoperfusion abnormalities may include lactic acidosis, oliguria and an acute alteration of mental status.

The concept of organ dysfunction is distinct from the notion of organ failure favoured by older epidemiological descriptions. Organ failure is a dichotomous event that is either present or absent. However, dynamically changing organ function is one the hallmarks of the septic syndrome. This is not taken into account when using the static criteria favoured by older epidemiological descriptions. Organ dysfunction is suggested to be a phenomenon whereby the organ is not capable of maintaining homeostasis. This dysfunction should be viewed as a continuum of change over time and as such seen as a dynamic process. However, criteria that are universally applicable in quantifying individual organ dysfunction were not proposed at that time. Investigators were still obliged, however, to have a set of criteria that they could use to assess the presence or absence of organ dysfunction. The following is a representative set of criteria which have been used to make this assessment in previous epidemiological studies (van Gestel et al., 2004).

Cardiovascular system dysfunction can be defined as a systolic blood pressure < 90mm Hg or mean arterial pressure < 60mm Hg or a reduction in blood pressure of more than 40mmHg from baseline measurements, in the absence of other causes for hypotension, despite adequate fluid resuscitation, or as the need to administer vasopressors or inotropes in order to maintain systolic blood pressure > 90mm Hg or mean arterial pressure > 60mm Hg.

Renal dysfunction may be defined as being present when urine output falls below 0.5ml/kg/hour for at least one hour, despite adequate fluid resuscitation, or when the concentration of serum creatinine climbs above 177mg/dL and this is not attributable to chronic renal failure.

Respiratory system dysfunction is usually said to be present when PaO2/FiO2 ratio is < 200mmHg in the presence of a respiratory infection or < 250mmHg if the infective source is other than pulmonary. It may also be indicated by the presence of assisted ventilation in the first 24 hours of ICU admission.

Central Nervous System (CNS) dysfunction is usually said to be present where there is an acute deterioration of the neurological condition, which is not attributable to the administration of sedation or to the presence of CNS disease.

The presence of a platelet count < 80,000 / mm^3 of blood, or a decrease of at least 50% in the 2 days preceding admission normally indicates haematological dysfunction.

Metabolic dysfunction is usually defined as the presence of a metabolic acidosis (pH < 7.30 or base deficit > 5mmol/l) in association with a plasma lactate level > 3mg/dL.

Hepatic dysfunction is normally said to be present seen when the bilirubin concentration rises above 43mg/dL, or alanine aminotransferase (ALT) levels exceed

50U or the International Normalised Ratio (INR) or the Activated Partial Thromboplastin Time (APTT) exceeds 1.5 times normal in the absence of systemic anticoagulant agents.

In the absence of clear guidance from the 1991 consensus conference some studies do vary slightly in their individual interpretation of organ dysfunction. The above broadly encompasses most definitions used and were also used in this study to define organ dysfunction.

Septic shock is another clearly defined point along the continuum of clinical severity seen in sepsis. It is defined as a subset of severe sepsis where cardiovascular dysfunction persists along with metabolic dysfunction despite adequate fluid resuscitation (1992). Patients receiving inotropes or vasopressors would still be considered to have septic shock despite the appearance of normal blood pressures.

1.3 Epidemiology

Sepsis remains an extremely prevalent disease, associated with a persistently high mortality rate, despite a vast increase in knowledge concerning the pathophysiology and the risk factors contributing to it. Incidence and mortality rates vary widely between studies and amongst different centers. The most recent large scale analysis of the epidemiology of severe sepsis in the US estimated the national annual incidence to be 3.0 cases per 1000 population (Angus et al., 2001). However, in a smaller European study of adult ICUs the incidence has been estimated at 0.54 cases per 1000 adults annually (van Gestel et al., 2004). The incidence demonstrates a pattern of increasing up to 100 fold with age. In the US it was observed to be high in infants (5.3/1000 aged <1year), deceased quickly in older children (0.2/1000 aged 5-14), increased slowly through most of adulthood (5.3/1000 aged 60-64), and increased sharply in the elderly (26.2/1000 aged >85) (Angus et al., 2001).

The average cost per patient has been estimated at $22,100 (Angus et al., 2001). Unsuprisingly, this figure increases for non-survivors, patients admitted to an ICU, surgical cases and increases proportionately with the number of organ failures.

The commonest source of infection identified in the American epidemiological study was the respiratory tract followed by the abdominal cavity (Angus et al., 2001). This pattern was also observed in a European study with a respiratory source observed in 47% of patients followed by an abdominal source in 34% (van Gestel et al., 2004). The commonest organ failure was shown to be the respiratory system (90%) followed by the cardiovascular system (72%) (van Gestel et al., 2004).

The mortality rate associated with sepsis remains stubbornly high, despite more than a decade of intensive research into potential therapies. Mortality rates can

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Metabolic dysfunction is usually defined as the presence of a metabolic acidosis (pH < 7.30 or base deficit > 5mmol/l) in association with a plasma lactate level > 3mg/dL.

Hepatic dysfunction is normally said to be present seen when the bilirubin concentration rises above 43mg/dL, or alanine aminotransferase (ALT) levels exceed

50U or the International Normalised Ratio (INR) or the Activated Partial Thromboplastin Time (APTT) exceeds 1.5 times normal in the absence of systemic anticoagulant agents.

In the absence of clear guidance from the 1991 consensus conference some studies do vary slightly in their individual interpretation of organ dysfunction. The above broadly encompasses most definitions used and were also used in this study to define organ dysfunction.

Septic shock is another clearly defined point along the continuum of clinical severity seen in sepsis. It is defined as a subset of severe sepsis where cardiovascular dysfunction persists along with metabolic dysfunction despite adequate fluid resuscitation (1992). Patients receiving inotropes or vasopressors would still be considered to have septic shock despite the appearance of normal blood pressures.

1.3 Epidemiology

Sepsis remains an extremely prevalent disease, associated with a persistently high mortality rate, despite a vast increase in knowledge concerning the pathophysiology and the risk factors contributing to it. Incidence and mortality rates vary widely between studies and amongst different centers. The most recent large scale analysis of the epidemiology of severe sepsis in the US estimated the national annual incidence to be 3.0 cases per 1000 population (Angus et al., 2001). However, in a smaller European study of adult ICUs the incidence has been estimated at 0.54 cases per 1000 adults annually (van Gestel et al., 2004). The incidence demonstrates a pattern of increasing up to 100 fold with age. In the US it was observed to be high in infants (5.3/1000 aged <1year), deceased quickly in older children (0.2/1000 aged 5-14), increased slowly through most of adulthood (5.3/1000 aged 60-64), and increased sharply in the elderly (26.2/1000 aged >85) (Angus et al., 2001).

The average cost per patient has been estimated at $22,100 (Angus et al., 2001). Unsuprisingly, this figure increases for non-survivors, patients admitted to an ICU, surgical cases and increases proportionately with the number of organ failures.

The commonest source of infection identified in the American epidemiological study was the respiratory tract followed by the abdominal cavity (Angus et al., 2001). This pattern was also observed in a European study with a respiratory source observed in 47% of patients followed by an abdominal source in 34% (van Gestel et al., 2004). The commonest organ failure was shown to be the respiratory system (90%) followed by the cardiovascular system (72%) (van Gestel et al., 2004).

The mortality rate associated with sepsis remains stubbornly high, despite more than a decade of intensive research into potential therapies. Mortality rates can

vary from 15% to 80% which may reflect the complex heterogeneity of sepsis (Angus and Wax, 2001; Martin et al., 2003). Mortality rates increase with pre-existing medical co-morbidities, with an increase in the number of failing organs, with increasing age and when the patient necessitates an ICU admission. Mortality rates can increase 3-fold when comparing children to those aged >85years (Angus et al., 2001). In a European context, the best estimate of mortality, at present, comes from a multi-center study among 170 French ICUs quoting a 28 day mortality rate of 56% in patients with severe sepsis (Brun-Buisson et al., 1995).

Regardless of the absolute figure, it is well recognised that severe sepsis is the most common cause of death in the non-coronary ICU (Niederman and Fein, 1990; Parrillo et al., 1990). To bring these statistics into perspective, it has been calculated that the number of people who died as a result of severe sepsis in the USA in 1995 was equal to the number of deaths after following myocardial infarctions for the same period (Angus et al., 2001).

This healthcare problem is predicted to worsen. It is estimated that the number of cases will increase at the rate of 1.5% per annum in the US (Angus et al., 2001), outstripping the anticipated population growth. This is, in part, due to the high prevalence of sepsis in the older population.

1.4 Overview of the pathophysiology of sepsis

Inflammation is the process by which the human body attempts to counteract and contain potentially injurious agents. This process is largely regulated by cytokines and chemokines. Inflammation in response to bacterial infection aims to enhance the bactericidal properties of a host and should facilitate clearance of the invading organism.

Cytokines are inducible proteins or glycoproteins, with a molecular mass of 8 to 30 kDa, which can be secreted by numerous cell types, and are used by organisms as signalling molecules. The main feature distinguishing them from endocrine hormones is their preference for acting at a local level. Thus cytokines usually act as paracrine or autocrine factors (Gol-Winkler, 1986; Henderson et al., 1996).

Chemokines, or chemotactic cytokines, are a large family of structurally homologous cytokines that stimulate leukocyte movement and regulate the migration of leukocytes from the blood to the tissues. Most chemokines are produced by a variety of cell types, in response to an inflammatory stimulus, and recruit leukocytes to sites of inflammation.

An inflammatory response may become harmful when unregulated. Bactericidal compounds, such as the reactive oxygen species, may become pathogenic when released in large quantities around normal tissue. In an attempt to prevent this, the inflammatory response is subject to multiple levels of control involving a diverse array of cell types and their soluble mediators. Consequently, a theory was popularised in the 1970s that envisaged invading micro organisms more as bystanders than as inherently pathogenic, with the main contributor to the organ damage observed during a septic episode being an intrinsic overly exuberant immune response that damages host tissue (Thomas, 1972). Until recently, the accepted interpretation

was that the clinical syndrome of sepsis results when the initial, appropriate host inflammatory response becomes amplified and uncontrolled and begins to damage the host tissues it was designed to protect (Bone et al., 1992; Stone, 1994; Warren, 1997). This interpretation has been based primarily on rodent or primate models of overwhelming endotoxaemia or bacteraemia. However, the response induced by an insult of this nature does not reflect the scenario observed in human sepsis (Deitch, 1998; Fink and Heard, 1990; O'Reilly et al., 1999). Animals stimulated in this manner do indeed exhibit an exuberant proinflammatory response with levels of circulating cytokines exponentially higher in these animals in comparison to humans with sepsis. Unsuprisingly, interventions that blocked these inflammatory mediators resulted in increased survival in these animal models (Deitch, 1998; Fink and Heard, 1990; O'Reilly et al., 1999). However, both the mode of administration and the quantity of administered stimuli may preclude any meaningful comparison with the situation observed in human sepsis.

An important observation, when interpreting the results of animal models, is the different responses observed when endotoxin and bacteria are used as stimuli. Using knockout mice, it has been demonstrated that loss of proinflammatory cytokine gene expression, whilst detrimental in combating an insult with live bacteria, is of benefit when the animals are stimulated with a sterile inflammatory stimulus such as endotoxin (dDharmana et al., 2002). The corollary to this in the human setting is the SIRS response to sterile and infective insults. Sterile inflammatory insults include pancreatitis, burns and acute transfusion related reactions. One of the best-defined sterile inflammatory reactions is that occurring following exposure to the extra-corporeal circuit utilised in cardiopulmonary bypass. Indeed, in prior experiments in this laboratory we have demonstrated that the inflammatory reaction, with associated

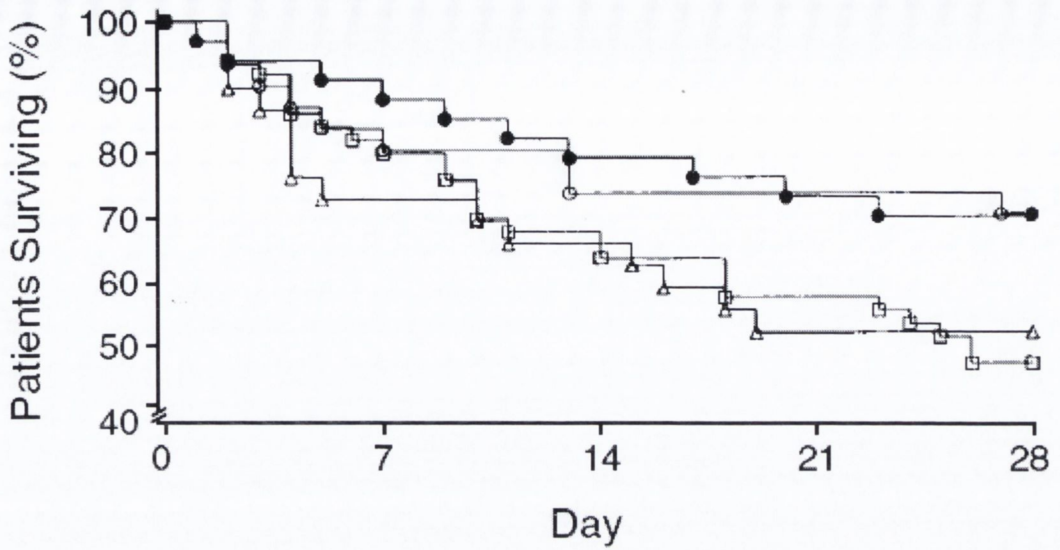
hypotension and lactic acidosis, invoked by this response is characterised by a relative excess of proinflammatory cytokines and a relative decrease in anti-inflammatory cytokines in comparison to patients that did not display a hypotensive acidotic response (Duggan et al., 2006).

It is likely, therefore, that at a cytokine production level, the mechanisms which culminate in a host becoming susceptible to and ultimately succumbing as a result of sepsis are different to that observed in either the human model of sterile inflammation or animal models utilising non-physiologically relevant doses of endotoxin or bacteria. This is despite similarities observed along the spectrum of these illnesses when evaluating them using purely clinical endpoints.

Indeed, it is plausible that the appropriate human immune response to infection may be dependant upon a vigorous proinflammatory response driven and propagated by proinflammatory cytokines. This inflammatory response may be necessary for adequate bactericidal activity in the host. There is support for this hypothesis from a combination of human and animal sources. Using a mouse model investigators have described a 100% mortality in response to infection with Salmonella bacteria in mice that have non functioning proinflammatory cytokine genes whereas there was a 0% mortality in wild type mice (Dharmana et al., 2002). Additionally, trials involving the treatment of septic patients with a blocking compound to the prototypical proinflammatory cytokine, tumor necrosis factor-alpha (TNFα), have been conducted with disappointing results (Abraham et al., 1997; Fisher et al., 1996; Fisher et al., 1993). Indeed, some of the results demonstrate a clear dose-response relationship between an increasing dose of the anti-TNFα compound, a resultant decrease in circulating physiologically available TNFα and an increasing mortality rate (Fisher et al., 1996) (figure 1.1). Furthermore, the failure of anti-

inflammatory therapy in human sepsis has been demonstrated in numerous trials of compounds that block the inflammatory response at various levels. This includes trials utilising corticosteroids, nonsteroidal anti-inflammatory drugs, anti-endotoxin agents and interleukin-1 receptor antagonists (Bernard et al., 1997; Bone et al., 1987; Fisher et al., 1994; Ziegler et al., 1991). Each of these trials was conducted based on the hypothesis that a proinflammatory response to infection is detrimental and is responsible for increased mortality in these patients. The failure of these therapies to demonstrate a benefit may lead one to the assumption that the hypothesis underlying these studies is inherently flawed.

Figure 1.1 (reproduced from (Fisher et al., 1996))



Study Group	No. of Patients	No. of Deaths
Placebo (-●-)	33	10
0.15 mg/kg (-○-)	30	9
0.45 mg/kg (-△-)	29	14
1.5 mg/kg (-□-)	49	26

Figure 1. Kaplan–Meier Analysis of Survival in Patients with Sepsis Receiving Placebo or One of Three Doses of TNFR:Fc. An intention-to-treat analysis of mortality from all causes at 28 days by the Cochran–Mantel–Haenszel test showed a dose-response relation between treatment with TNFR:Fc and mortality (P = 0.02). Mortality did not differ significantly between the placebo group and the three treatment groups combined (P = 0.13).

Furthermore, there is recent evidence, from animal models and *ex vivo* cell stimulation experiments, demonstrating the beneficial effects of proinflammatory cytokines in sepsis. Animal models of peritonitis utilising TNFα blocking agents have been shown to decrease survival (Echtenacher et al., 2001; Eskandari et al., 1992). Using a "two-hit" model of sepsis, investigators demonstrated that a bacterial challenge that was readily cleared in other groups, resulted in the peripheral seeding of bacterial species in a group that had been subjected to cecal ligation and puncture

(CPL) 5 days previously (Murphey et al., 2004). The impaired production of cytokines crucial to the initiation and propagation of effector immune cell functions characterised the response of these animals to bacterial challenge. A further animal model demonstrated that in late murine sepsis, the proinflammatory T helper cell type 1 (Th1) response was decreased with development of an anti-inflammatory Th2 phenotype (Ono et al., 2001). Furthermore, therapies designed to augment the production of Th1 cytokines demonstrated a survival benefit in this model.

That these more recent animal models of sepsis directly contradict the findings of some of the earlier studies is likely a reflection of the different models used. That there are so many models of sepsis and septic shock is tacit evidence that none of them are perfect. However, as most deaths in an adult ICU occur after a protracted course there is a need for an animal model of severe chronic sepsis. The murine model of CLP used in the studies referenced above, despite its shortcomings, may come closest to reflecting this as the animals can survive for up to 1 week thereby displaying features similar to that observed in late sepsis in humans.

Finally, immune cells isolated from patients after either a septic or traumatic episode frequently display features of immunoparalysis, characterised by deficient IL-12 and IFNγ production with retention of the ability to produce IL-10 (Goebel et al., 2000; Nakos et al., 2002; Rigato and Salomao, 2003). Furthermore, treatment of septic patients with proinflammatory compounds, such as IFNγ, has shown promise both in reversing the immunoparalysis and in terms of patient morbidity (Docke et al., 1997; Nakos et al., 2002).

Despite this evidence, it is unlikely that the use of any one compound in sepsis will be of benefit to all patients due to the heterogenous nature of the disease. This may explain why, despite the overall poor results obtained with TNFα antagonists,

some subgroups did demonstrate a survival advantage (Fisher et al., 1996; Reinhart and Karzai, 2001). A meta-analysis of clinical trials of anti-inflammatory agents in sepsis showed that although high dose anti-inflammatory treatment is harmful in general, there is a subgroup that benefit from this treatment (Zeni et al., 1997). Additionally, despite encouraging case series data of the use of IFNγ in patients with a demonstrable immune deficiency, multicenter trials are yet to describe an overall survival benefit (Polk et al., 1992; Wasserman et al., 1998). These aberrant results are possibly explained by a small subgroup of septic patients with greatly exaggerated levels of circulating proinflammatory cytokines, which contribute to excess morbidity through the mechanisms postulated earlier (Girardin et al., 1988; Hatherill et al., 2000). Thus, given the heterogenous nature of the molecular basis of this disease, what may prove to be of benefit ultimately is the individual treatment of patients, based on their specific immune response, in a timely and targeted fashion.

The ultimate cause of death in patients with sepsis is usually difficult to define. Autopsy studies do not reveal the exact cause of death in sepsis (Hotchkiss and Karl, 2003). Due primarily to effective organ support therapy, it is rare that a patient would die secondary to refractory shock or hypoxia, despite the frequency of septic shock and the acute respiratory distress syndrome in these patients. Renal failure is common but dialysis can indefinitely support kidney function in an ICU setting. Most patients die when care is withdrawn or capped at a predefined level when a decision is reached that further treatment is futile. This associated organ failure has been attributed to both disseminated coagulopathy and to disruption of the integrity of micro-vascular endothelium (Cohen, 2002). It is additionally plausible that ongoing bacterial overgrowth contributes to organ failure or that organ compromise is mediated via a cytokine response that contributes to inflammation,

whilst not being adequately bactericidal. Evidence for this latter scenario comes from an animal model of pneumonia that describes adequate leukocyte recruitment to the area of infection yet there is a quantitative lack of adequate bactericidal compounds being produced (Deng et al., 2004).

Whatever the molecular mechanisms involved, at a systemic level patients will typically develop a single organ failure and if the disease progresses, patients will develop failure of other organ systems. If four or five organs fail the mortality is greater than 90%, irrespective of treatment (Cohen, 2002).

Respiratory failure is common and usually progresses rapidly. A sustained respiratory rate that exceeds 30 breaths per minute is a sign of impending ventilatory collapse. Timely intubation and mechanical ventilation reduce respiratory oxygen demand and the risk of aspiration and cerebral anoxia from catastrophic respiratory arrest. Nearly 85% of patients require ventilatory support, typically for 7-14 days, and almost half of these patients meet the criteria for the diagnosis of the acute respiratory distress syndrome (Bernard et al., 1997; Wheeler and Bernard, 1999).

Cardiovascular failure is manifested by shock. This results in an inadequate supply of oxygen, resulting in lactic acidosis and tissue damage. Circulatory adequacy is best gauged using several indices, including mentation, urinary output, skin perfusion and blood pressure. Use of blood pressure alone is problematic, because the administration of vasoactive drugs and fluids can normalise blood pressure without correcting the fundamental defect. In sepsis hypotension occurs as a result of failure of the vascular smooth muscle to constrict. This vasodilatory shock is characterised by peripheral vasodilation and a poor response to therapy with vasopressor drugs. At least three mechanisms have been implicated as being causal; these are, activation of ATP-sensitive potassium channels in the plasma membrane of vascular smooth

muscle, activation of the inducible form of nitric oxide synthase and deficiency of the hormone vasopressin (Landry and Oliver, 2001). Also present is a reduction in myocardial contractility which stems from the numerous circulating myocardial depressant factors, often partly correcting by additional beta-adrenergic stimulation (Bunnell and Parrillo, 1996).

Renal failure requiring dialysis occurs in fewer than 5% of patients (Bernard et al., 1997; Wheeler and Bernard, 1999). However, transient oliguria and moderate increases in serum creatinine is common. This is usually related to hypotension and restoration of normal perfusion normally reverses the oliguria.

Frank hepatic failure is very uncommon in sepsis. Conversely, abnormalities in serum aminotransferase and bilirubin levels are common.

The cumulative effects of hypotension, hypoxaemia and treatment with sedatives are responsible for most changes in mentation. As a result consciousness is frequently altered in patients with sepsis. Substantial reductions in scores on objective scales of neurological function that are not due to medication portend a dismal prognosis and are usually the result of anoxia or intracranial haemorrhage (Bastos et al., 1993; Sprung et al., 1990).

In summary, the hypothesis surrounding the pathophysiology of sepsis on a molecular level has changed over time from one defining a pathogenically exuberant proinflammatory response damaging host organ tissue to that of a deficient immune/ inflammatory response leading to a subsequent failure to clear the infecting organism. As a consequence of the heterogenous nature of the sepsis response, it is possible that both scenarios exist in different patients. Organ failure may ensue from either scenario, with the likelihood of death increasing exponentially for each additional organ system failure.

1.5 Immunity and T helper cell development

1.5.1 Innate and adaptive immunity

Defence against invading microbes is mediated by the early reactions of innate immunity and the later responses of adaptive immunity. Innate immunity consists of cellular defence mechanisms that are in place prior to infection and do not require priming by a previous infection to be effective. The innate immune response reacts to structures that are common between groups of related microbes and is a non-specific type of immunity.

Adaptive immunity, however, is stimulated by exposure to infectious agents and increases in magnitude with each successive exposure to a particular microbe. The cardinal features of the adaptive immune response include; specificity for distinct antigenic molecules and the ability to remember exposure to a particular microbe and respond more vigorously to repeated exposures by the same microbe.

The adaptive response can be divided into two categories; humoral and cell-mediated immunity. Humoral immunity is mediated by antibodies, which target microbes for elimination. It functions primarily against extracellular microbes and their toxins. Cell-mediated immunity, on the other hand, is mediated by T lymphocytes and targets primarily intra-cellular pathogens.

Effective host defence requires the coordinated response of both innate and adaptive immunity. The two responses are not distinct, however, and the innate response can stimulate and influence the nature of the adaptive responses. In turn, the adaptive response utilises many of the effector mechanisms of the innate response to eliminate microbes. Several cytokines that are produced as components of innate immunity, such as IL-18, IL-12 and IL-10, can directly regulate the pattern of the

specific cell mediated response by influencing the growth and differentiation of T cells (Trinchieri and Scott, 1995).

1.5.2 Tumor necrosis factor-alpha

Encoded on the short arm of chromosome 6, TNFα plays a critical role in regulating the innate immune system whilst also being key to the development and propagation of acquired immune responses.

Produced primarily by activated macrophages, TNFα production is induced by lipopolysaccaride (LPS), IFNγ and other inflammatory cytokines including the recently identified IL-23 and IL-17 (Hunter, 2005; Vassalli, 1992). TNFα, in turn, stimulates a Th1 response thus making it a mediator of both innate and adaptive immunity and therefore an important link between specific immune responses and acute inflammation. Other cells with the potential to produce TNFα are T lymphocytes, polymorphonuclear leukocytes (PMN), mast cells, smooth muscle cells and tumor cells (Steffen et al., 1988; Vassalli, 1992). As the TNFα receptor is present on almost all nucleated cells, it has profound systemic effects. A positive feedback cycle ensures that TNFα induces its own synthesis and release (Philip and Epstein, 1986). This is in part achieved through TNFα mediated induction of NFκB transcription which in turn enhances the transcription of the TNFα gene (Collart et al., 1990; Shakhov et al., 1990).

The main source of TNFα, activated mononuclear phagocytes, are also one of its main targets. TNFα induces activation and differentiation of monocytes, is chemotactic for monocytes in vitro and induces a self amplifying chain of events leading to more TNFα release (Vassalli, 1992). These actions are strongly synergised by interferon gamma (IFNγ). Macrophage activation results in induction of two

independent cytotoxic pathways; the superoxide pathway and the nitric oxide pathway. Induction of these pathways explains the marked bactericidal effects of TNFα.

Critical to local inflammatory responses is the action of TNFα on endothelial cells. TNFα causes vascular endothelial cells to express adhesion molecules for leukocytes and induces the release of IL-6 and the potent chemotactic agent IL-8 (Gimbrone et al., 1989; Jirik et al., 1989; Vassalli, 1992). The administration of high doses of TNFα reproduces some of the characteristics of septic shock. Hypotension, from impaired myocardial contractility and reduced vascular smooth muscle tone, acidosis, hepatic necrosis, a coagulopathy and ARDS-like pulmonary changes are constant findings with injection of high doses of TNFα (Vassalli, 1992). Conversely however, the essential protective effects of TNFα against lethal bacterial challenge have been also demonstrated (Hunter et al., 1996; Silva et al., 1995). This a consequence of TNFα's ability to induce critical host defence mechanisms, including the generation of cytotoxic oxygen products (Silva et al., 1995).

In summary, TNFα has varied and wide ranging effects. It can activate and promote differentiation of macrophages, prompt neutrophils to degranulate and release more cytokines and stimulate activated lymphocytes to divide. It triggers the release of numerous mediators of inflammation. It facilitates the local accumulation of it's best producers, macrophages, and enhances their ability to attack microbes by inducing the production of reactive oxygen and NO derivatives. It also provides a critical link between the innate and adaptive immune response.

1.5.3 T helper cell differentiation

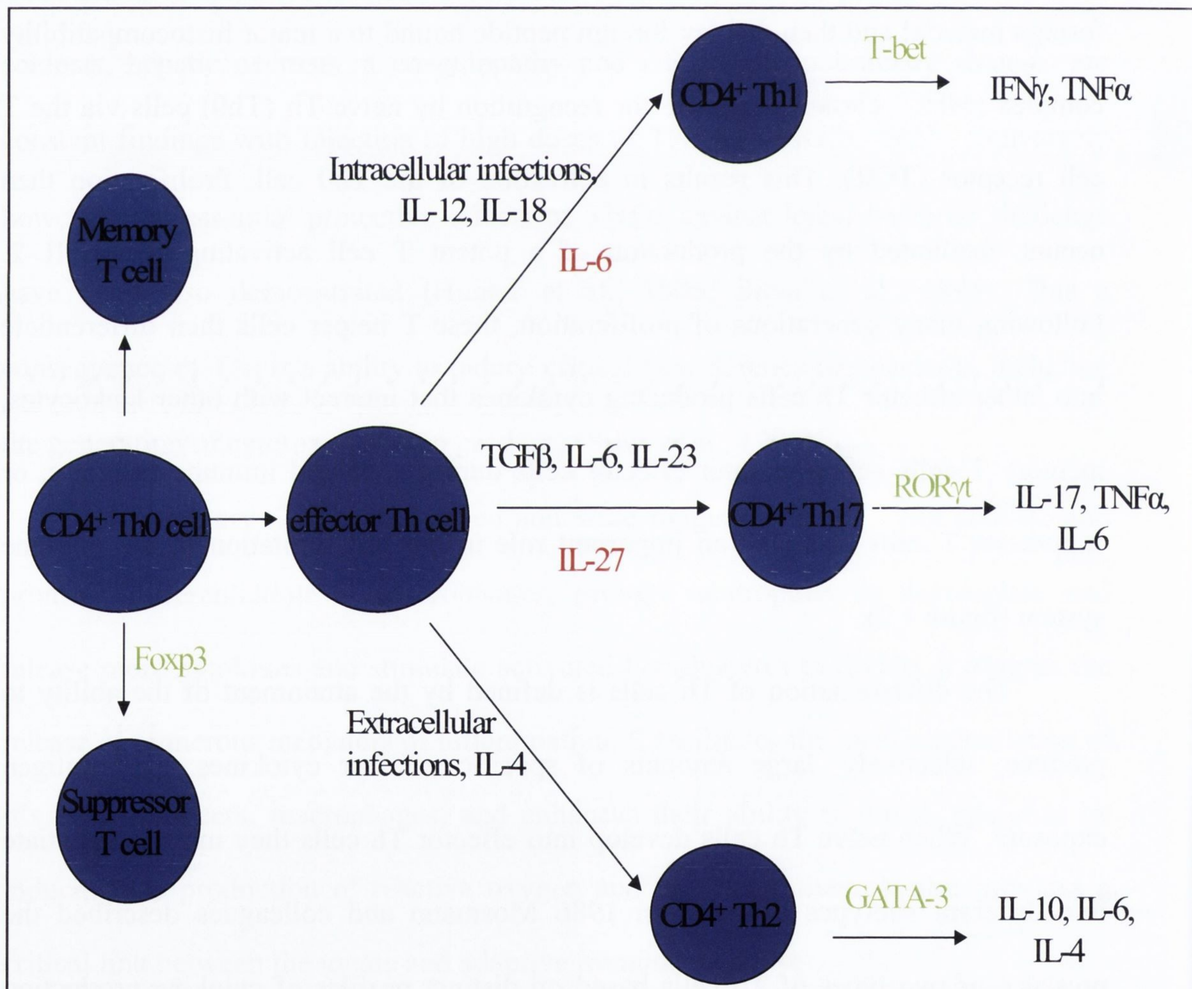
T helper (Th) cells are a subgroup of lymphocytes that play a critical role in regulating the bactericidal capabilities of the immune system. Interestingly, these cells display no inherent cytotoxic or phagocytic properties themselves but act in cooperation with other effector immune cells. In particular, Th cells play a role in humoral immunity by determining B cell antibody class switching and also play a role in cell mediated immunity by maximising the bactericidal activity of cytotoxic T cells and phagocytes.

During an immune response, antigen presenting cells (APCs) endocytose foreign material and then display foreign peptide bound to a major histocompatibility complex (MHC) class 2 molecule for recognition by naïve Th (Th0) cells via the T cell receptor (TCR). This results in activation of the Th0 cell. Proliferation then occurs, facilitated by the production of a potent T cell activating factor, IL-2. Following many generations of proliferation, these T helper cells then differentiate into either effector Th cells producing cytokines that interact with other leukocytes, memory T cells acting as later effector cells during a second immune response, or suppressor T cells that play an important role in the self limitation of the immune system (figure 1.2).

The differentiation of Th cells is defined by the attainment of the ability to produce, selectively, large amounts of specific effector cytokines upon antigen exposure. When naïve Th cells develop into effector Th cells they may differentiate into different subtypes of cells. In 1986 Mosmann and colleagues described the presence of two types of Th cells based on distinct profiles of cytokine production (Mosmann et al., 1986). IFNγ, TNFα and TNFβ are among the cytokines produced by the Th1 response, which utilise the transcription factor T-bet. In contrast, the Th 2

response is defined by the production of cytokines such as IL-4, IL-6 and IL-10, and the transcription factor GATA-3. The most important factor governing the polarisation of Th 0 cells into the appropriate Th 1 or Th 2 response is the local cytokine environment to which the Th 0 cell is exposed (Szabo et al., 2003). Other factors include the dose of antigen and the source of co-stimulation. This dichotomy provides a rational explanation for the distinct immune responses observed with cell-mediated and humoral immunity.

Figure 1.2



Schematic representation of the factors affecting the maturation of naïve T cells into effector Th cells. Positive regulators are shown in black, negative regulators are shown in red and transcription factors are shown in green.

1.5.4 The T helper cell type 2 response and Interleukin 4

Th 2 biased responses are classically responsible for the eradication of extracellular organisms such as helminthic and arthropod infections. *In vitro* studies suggest that Th 2 differentiation occurs through the activation of naïve T cells mediated by TCR crosslinking in the presence of IL-4 (Ansel et al., 2006). The main cellular sources of IL-4 include mast cells, basophils, eosinphils, natural killer T cells and previously differentiated Th2 cells. The receptor for IL-4 is present on naïve T cells and receptor occupancy induces STAT6 (signal transduction and activtor of transcription 6) activation along with expression of the transcription factor GATA3, which is a major regulator of Th 2 lineage commitment. Subsequent mature Th2 cells preferentially secrete a number of cytokines including IL-4 itself, IL-10 and IL-6. These cytokines stimulate proliferation of B cells and are considered necessary for the full maturation of the humoral immune system. The antibodies stimulated by this response, however, do not promote phagocytosis or activate complement efficiently. This situation may lead to chronic T cell stimulation often without a significant innate immune response or macrophage activation. Following initial priming with IL-4, Th2 cells subsequently become resistant to Th 1 polarising cytokines and IL-4 production then becomes independent of extrinsic IL-4 (Murphy et al., 2000).

1.5.5 Interleukin 10

IL-10, originally known as cytokine synthesis inhibitory factor, was initially found to be produced by stimulated Th 2 cells where it functioned by inhibiting cytokine production by Th 1 cells (Fiorentino et al., 1989). However, it is now recognised that IL-10 is produced by a wide variety of cells, including suppressor T

cells and macrophages. IL-10 enhances survival of normal human B cells and induces proliferation of activated B cells in concert with IL-4 (Moore et al., 2001).

Activation of the IL-10 receptor complex results in the inhibition of synthesis of several cytokines and in the reduction of their activities on their target cells. IL-10 activates the suppressor of cytokine signalling-1 (SOCS-1) and SOC-3 genes (Ding et al., 2003). SOCS-1 inhibits IFNγ, IL-4 and IL-10 induced signal transduction (Yasukawa et al., 1999) while SOCS-3 can inhibit signalling initiated by IL-6 (Niemand et al., 2003). The other main anti-inflammatory pathway employed by IL-10 is inhibition of NFκβ activation by inhibiting Iκβ kinase (IKK) (Schottelius et al., 1999). Inhibition of IKK prevents NFκB and the IκB family of proteins from dissociating, thereby preventing nuclear import and export of NFκB.

In human macrophages, IL-10 has the effect of inhibiting gene expression of most inducible cytokines and chemokines (Moore et al., 2001). The inhibitory effects of IL-10 on TNFα and IL-1 are crucial to its anti-inflammatory properties as these cytokines act synergistically on many inflammatory pathways and induce many secondary mediators such as chemokines. IL-10 inhibits expression of MHC class II antigens on monocytes thus affecting the T cell activating capacity of monocyte APCs (Moore et al., 2001). IL-10 acts on dendritic cells (DC) to produce a response consistent with inhibition of Th1 inflammatory responses (Moore et al., 2001). The potential beneficial effect of this inhibition of production of chemokines and proinflammatory cytokines and mediators is that it is likely to limit the duration and potential harmful pathology of inflammatory responses.

IL-10 plays a central role in striking a balance between pathology and protection and is central to the process of containment and eventual termination of

inflammatory responses. In doing so IL-10 facilitates elimination of infectious organisms with minimal damage to host tissues.

1.5.6 Interleukin 6

The cytokine IL-6 is expressed by several cell types, including mononuclear phagocytes, vascular endothelial cells, dendritic cells, T and B lymphocytes and fibroblasts, in response to variety of external stimuli such as microbes and other cytokines, including IL-4, IL-1, and TNFα (Kishimoto, 2005). By stimulating the synthesis of acute-phase proteins by hepatocytes, IL-6 contributes to the systemic effects of inflammation. Furthermore, by stimulating the production of neutrophils and by inducing the terminal differentiation of B cells to antibody producing plasma cells, IL-6 performs an important role in the link between innate and adaptive immunity (Hirano, 1998).

A novel function has recently been ascribed to IL-6 in the maturation of naïve Th cells (Diehl and Rincon, 2002). Two independent pathways involving IL-6 mediate propagation of a Th 2 response. IL-6 leads to the production of endogenous IL-4 culminating in a Th 2 response whilst also inhibiting Th1 differentiation by upregulating SOCS-1 gene expression in an IL-4 independent manner.

Therefore, IL-6 is a pleiotropic cytokine with diverse inflammatory properties and also possesses an ability to influence T cell responses through the preferential development of a Th 2 phenotype.

1.5.7 The T helper cell type 1 response and Interleukin 12

The Th 1 cellular response is generally induced by, and particularly effective against, intracellular pathogens and those that activate macrophages and NK cells. A

Th 1 response is associated with enhanced cell-mediated immunity and is therefore the appropriate response expected in the face of most common septic insults that result in ICU admission in the developed world. Lymphocytes demonstrating a Th 1 response pattern characteristically produce IFNγ. Requisite for generating optimal Th 1 responses in many experimental settings is the presence of IL-12 which activates STAT6 (Szabo et al., 2003). The resultant IFNγ then activates the STAT1 transcription factor and leads to subsequent T-bet expression, both of which are also essential for Th 1 development.

IL-12 is a heterodimeric cytokine that is produced, in response to stimulation, primarily by APCs, but also by neutrophils and macrophages and is composed of two disulfide-linked subunits designated p35 and p40 which are encoded by distinct genes (Gately et al., 1998). Secretion of IL-12 by macrophages directly induces IFNγ gene transcription in susceptible cell types and is directly inhibited by the cytokine products of Th 2 cells (Boehm et al., 1997; Lederer et al., 1996). The most distinctive of IL-12's activities is its ability to regulate the balance between Th 1 and Th 2 cells. This it achieves by promoting the differentiation of naïve T cells into a population of Th 1 cells on exposure to antigen. Furthermore, it serves as a co-stimulus required for maximum secretion of IFNγ by differentiated Th 1 cells and it also stimulates the development of Th1 cells from resting memory T cells on repeat exposure to antigen. Consequently, the beneficial role of IL-12 in promoting resistance to a variety of intracellular pathogens is well described (Trinchieri and Scott, 1995).

The IL-12 receptor is primarily expressed on activated T and NK cells, where it acts to increase their lytic activity whilst also acting as a short-term growth factor. It has been shown that the IL-12 receptor β2 subunit is not expressed on differentiated

Th 2 cells, providing a mechanism for regulating IL-12 responsiveness (Rogge et al., 1997; Szabo et al., 1997).

IL-12, therefore, is an important link between innate and adaptive immunity. It is produced during early innate immune reactions against intracellular microbes and stimulates an adaptive immune response that protects the host against these microbes.

1.5.8 Interferon gamma

The main cellular sources of IFNγ are activated NK cells (Perussia, 1991), activated Th 1 cells (Mosmann and Coffman, 1989) and activated $CD8^+$ cytotoxic cells (Boehm et al., 1997). IFNγ production is primarily stimulated by IL-12, TNFα and IFNγ itself (Hardy and Sawada, 1989). The IFNγ receptor is expressed on most nucleated cells at modest levels.

Several primary response genes are themselves transcription factors and are required for the induction of the other secondary components of the cellular response to IFNγ. One of these transcription factors, IFNγ regulatory factor-γ, is also regulated by TNFα, and is consequently partly responsible for the synergism observed between IFNγ and TNFα (Boehm et al., 1997).

The primary determinant of Th 1 differentiation is IL-12, by directly inducing IFNγ secretion in both naïve $CD4^+$ T cells and NK cells (Trinchieri and Scott, 1995). There is also a positive feedback loop whereby IFNγ in turn induces IL-12 secretion from monocytes leading some commentators to label IFNγ as an inducer of the Th 1 phenotype (Seder, 1994; Trinchieri and Scott, 1995).

IFNγ enhances MHC-associated antigen presentation (Farrar and Schreiber, 1993) and also promotes T lymphocyte and macrophage endothelial adhesion and extravasation to sites of infection. This promotes the local formation of inflammatory

exudates enriched in the agents of cellular immunity (Boehm et al., 1997). Macrophage activation, through induction of nitric oxide and superoxide production, is another major endpoint of IFNγ production. This is of critical importance in the elimination of intracellular organisms. Similar changes occur in neutrophils.

IFNγ also acts on B cells to promote switching to certain IgG subclasses and inhibits switching to IL-4 dependant isotypes. This induces antibody responses that also participate in phagocyte–mediated elimination of microbes, in concert with the direct macrophage-activating effects of this cytokine.

The net effect of these properties of IFNγ is the implementation of the Th 1 response with the promotion of macrophage-rich inflammatory reactions, while inhibiting IgE dependant eosinophil-rich reactions.

1.5.9 Interleukin 18

Produced primarily by macrophages and dendritic cells, IL-18, in cooperation with IL-12, enhances the release of IFNγ, from effector Th1 cells. Whilst not essential for Th 1 differentiation, IL-18 facilitates IL-12 directed Th1 differentiation through optimisation of IFNγ production (Szabo et al., 2003). The IL-18 receptor is also expressed on NK cells, macrophages and endothelial cells and functions through the promotion of nuclear translocation of NFκB (Tschoeke et al., 2006). Although the pleiotropic effects of IL-18 have suggested a role in modulating the Th 2 immune response depending on the local cytokine environment (Leite-De-Moraes et al., 2001; Xu et al., 2000), the most prominent effect of increased IL-18 is recognised to be the promotion of proinflammatory Th 1 cytokine production.

1.5.10 An overlap between the T helper cell type 1 response and the T helper cell type 17 response and the roles of Interleukin 23 and Interleukin 27

The recent discovery of a subset of $CD4^+$ T cells that produces members of the IL-17 family of cytokines, TNFα and IL-6 disrupted the simple dichotomy of the Th cell classification by adding a Th 17 subset of T cells (Aggarwal et al., 2003). This Th 17 lineage of T cells is purported to be of critical importance both in driving an autoimmune inflammatory response, a property previously attributed to the Th 1 response, and also in host protection against bacterial infection (Hunter, 2005; Ye et al., 2001). It has been demonstrated that Th17 cells develop from naïve T cells following stimulation with transforming growth factor-β and IL-6 and these cells utilise the transcription factor RORγt (Bettelli et al., 2006; Veldhoen et al., 2006).

Critical to the development of this T cell lineage is IL-23, which is a heterodimer composed of a specific p19 subunit and the same p40 subunit which is also a component of IL-12. Produced by activated dendritic cells and macrophages, IL-23 was originally thought to mediate similar biological functions to IL-12 (Szabo et al., 2003). However, it has recently been appreciated that that IL-23 in fact plays a limited role in promoting classic cell mediated immunity and functions primarily to stimulate T cells to produce IL-17. Interestingly, the IL-23 receptor is not present on naïve T cells but is detectable on memory $CD4^+$ T cells (Parham et al., 2002). This suggests that IL-23 may be preferentially involved in sustaining cytokine production in the later stages of inflammation rather than initiating a pro inflammatory response development from naïve $CD4^+$ T cells. IL-23 may be of particular importance in the production of TNFα as it has the capacity to stimulate its production via two independent pathways. TNFα production may be induced by IL-17 with IL-23 as a

positive regulator of this response whilst additionally acting directly on macrophages to stimulate TNFα production (Hunter, 2005; Lubberts et al., 2005).

IL-27 is another homolog of IL-12, which has been implicated in the regulation of T cell development. Its precise function has remained somewhat more elusive than was the case for IL-23 and it was initially labelled as an inducer of Th1 differentiation and proliferation (Szabo et al., 2003). However, it has recently been shown to be involved in the repression of the development of Th17 cells (Batten et al., 2006; Stumhofer et al., 2006). These studies demonstrated that IL-27 is a potent suppressor of Th17 cell development via a STAT 1 dependant process and that in contrast to IL-23, it appears that a predominant function of IL-27 is to limit the extent of the innate and adaptive immune responses.

These recent discoveries have added to our understanding of the complexities surrounding Th cell development. Whilst undoubtedly incomplete, this improved appreciation allows us to now attribute certain autoimmune disorders that did not fit into the Th1/Th2 paradigm to alterations of this novel subset of Th17 cells.

1.6 The role of nitric oxide and asymmetrical dimethyl arginine in sepsis

Endothelium derived nitric oxide (NO) is a potent vasodilator which antagonises the effects of endogenous vasopressors (Cooke and Dzau, 1997). NO is produced from L-arginine by an enzyme, Nitric Oxide Synthase (NOS), which exists in constitutive, inducible (i), endothelial (e) and neuronal (n) isoforms. The endothelial isoform (eNOS) regulates vascular tone and interactions between leukocytes and endothelium (Nijveldt et al., 2003a). Consequently, NO has been implicated in the pathogenesis of the hypotension and organ failure attributable to severe sepsis (Landry and Oliver, 2001). However, although non selective pharmacologic inhibition of NOS briefly attenuates the haemodynamic anomalies seen in these patients with severe sepsis, the overall effect of such inhibition is to increase mortality (Lopez et al., 2004).

This conundrum may, in part, be explained either by the selective inhibition of the various isoforms of NOS or by an ancillary non-vascular function of NOS. On the one hand, inhibition of the constitutively expressed isoform of NOS, essential to maintain organ perfusion, may be detrimental (Nijveldt et al., 2003b) and may be contribute to the increased mortality observed in these studies. Of considerably greater relevance in the context of sepsis are NO's ancillary protective functions. NO is known to possess potent anti-microbial properties, antagonism of which may contribute to the excess mortality observed with NOS inhibition in patients with sepsis (Boehm et al., 1997). Indeed, many of the cytokines involved in the inflammatory response to infection function by upregulating transcription of NOS, leading ultimately to an enhanced bactericidal state.

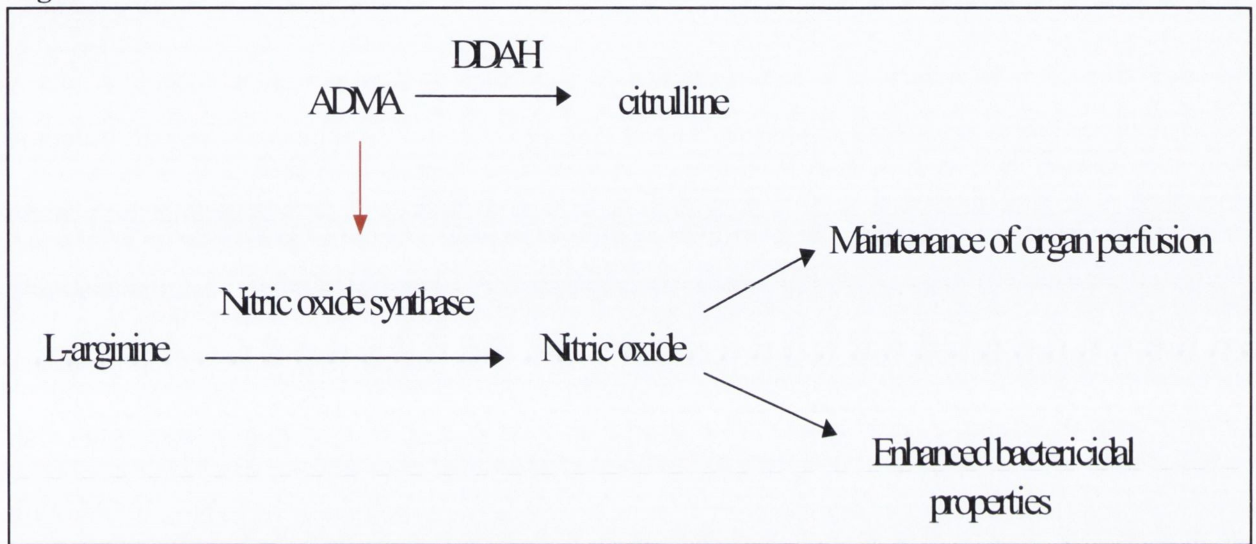
Asymmetrical dimethyl arginine (ADMA) is a naturally occurring non selective inhibitor of NOS, derived from protein catabolism, and is metabolised to

citrulline by dimethylarginine dimethylaminohydrolase (DDAH) (Tran et al., 2003). This enzyme exists as two isoforms, with DDAH II found primarily in tissues that express eNOS. Notably, DDAH displays reduced activity when operating in an inflammatory milieu (Ito et al., 1999). The co-localisation of DDAH and NOS at several sites supports the hypothesis that DDAH may regulate NOS activity by controlling the metabolism of ADMA (Tran et al., 2003).

NO depletion by ADMA is likely to have biologic significance, as elevated ADMA levels are seen in patients with vascular disease, hepatic failure and renal failure, and are linked with greater severity of organ failure in ICU patients with sepsis (Nijveldt et al., 2003a; Siroen et al., 2005). Furthermore, following a landmark paper demonstrating the beneficial effects of intensive insulin therapy in the ICU (Van den Berghe et al., 2006), this group subsequently postulated that the beneficial effects of the administration of exogenous insulin may be associated with an ability of insulin to regulate ADMA levels in septic patients (Siroen et al., 2005). However, additional mechanisms are obviously at work, as this group demonstrated that only a minority of the variability in ADMA levels is explained by variation in insulin levels. It is plausible that variation in ADMA levels may also have an additional genetic component. Several polymorphisms have been documented in the *DDAH II* gene, at least one of which, located in the promoter region, has been associated with altered expression of DDAH II (Jones et al., 2003). This in turn could plausibly affect ADMA levels. However, these polymorphisms have not been studied in a human septic population previously. Interestingly, the association that we have previously described in this laboratory between polymorphism in the promoter region of the *DDAH II* gene and systemic arterial vasodilation following cardiac surgery with

cardiopulmonary bypass, suggests a link between pathologic vasodilation, such as occurs with severe sepsis, and ADMA metabolism (Ryan et al., 2006).

Figure 1.3



Schematic representation of the interactions between the NOS pathway and the ADMA metabolic pathway. The red line indicates an inhibitory effect.

1.7 The role of genetic variability in sepsis

Although there is a clear genetic susceptibility to premature death from sepsis, (Sorensen et al., 1988) elucidation of the causative genes has proven a more intractable problem. The search for candidate genes continues unabated, as there are clear advantages in being able to identify susceptible patients through genotyping prior to exposure to potential septic insults.

TNFα was heralded as a candidate gene in the susceptibility to, and outcome from human sepsis as a consequence of early animal models implicating the protein product as pathogenic in this setting (Tracey et al., 1986). The most exhaustively researched genetic variant in septic cohorts, in the TNFα gene, is a promoter polymorphism at position –308. Whilst not consistently reproducible, the studies that do describe an association at this locus find that the presence of the TNF2 allele, with an adenine (A) at position –308, to be predictive of susceptibility to sepsis and an increased mortality from a septic insult (Clark and Baudouin, 2006). Additionally, SNPs at positions –238, -857 and -863 have also been associated with aberrant inflammatory responses in some studies (Rahman et al., 2006).

Functional studies examining the influence of the TNFα -308 single nucleotide polymorphism (SNP) on gene expression have provided conflicting and inconsistent results (Appoloni et al., 2001; Stuber et al., 1995). An ELISA based technique, quantifying circulating protein levels, remains the most popular surrogate for gene expression in this setting. However, ELISA has been proven to lack adequate sensitivity when quantifying cytokines in a clinical setting with some studies demonstrating that only 10% of septic patients have detectable cytokine protein (Pruitt et al., 1996). Consequently, functional studies have relied on amplified TNFα gene expression using a stimulus such as LPS in order to generate sufficient quantities

of protein. This intervention obviously introduces the possibility of misinterpretation of these results. As an alternative, mRNA quantification may be a more appropriate method for quantifying gene expression as protein levels may be influenced by post-transcriptional regulation unrelated to genetic polymorphisms. The exquisite sensitivity of this technique in inflammatory states has previously been demonstrated (Duggan et al., 2006; Pachot et al., 2005a; Pachot et al., 2005b).

Another cytokine that has been extensively investigated previously in genotyping studies in the setting of sepsis is IL-6. Plasma concentrations of IL-6 have been associated with mortality from sepsis (Fisher et al., 1993) and reporter gene assays have demonstrated that the presence of a C allele at position –174 in the IL-6 promoter is associated with decreased IL-6 production whilst C homozygotes have the lowest basal levels of circulating plasma IL-6 in healthy subjects (Fishman et al., 1998). However, the –174 SNP has not been conclusively linked to sepsis susceptibility and outcome, as genetic association studies have thus far produced conflicting results (Lin and Albertson, 2004).

High plasma concentrations of plasminogen activator inhibitor-1 (PAI-1) have also been associated with poor outcome in sepsis (Paramo et al., 1990). Additionally, the 4G allele of the 4G/5G insertion/deletion promoter polymorphism has been associated with higher plasma PAI-1 concentration (Eriksson et al., 1995). Studies have supported an association between PAI-1 polymorphism and poor outcome in meningococcal or post-trauma sepsis (Lin and Albertson, 2004). However, the data for sepsis susceptibility and outcome outside of these groups remain unclear.

1.8 The role of the inhibitor of kappa B-like gene in sepsis

An emerging candidate gene in the susceptibility to and the outcome from sepsis is the inhibitor of kappa B-like gene (IκBL). Suspicion surrounds this gene for two main reasons; its' genomic location and its' purported function.

IκBL is located telomeric of the TNF cluster in the central MHC on chromosome 6 (Albertella and Campbell, 1994). The MHC encodes for the human leukocyte antigen class I and class II molecules, which play an important role in adaptive immunity. Between these 2 gene clusters is a region densely packed with a selection of genes involved in a variety biological activities predominantly related to immunity. These include *TNFα*, *lymphotoxin alpha* and *DDAH II* amongst others. *IκBL's* genomic location, in the midst of these inflammatory-related cluster of genes, may be indicative of a common function attributable to these genes. Furthermore, polymorphisms in the promoter region of the IκBL gene have previously been associated with inflammatory disorders and altered regulation of the gene (de la Concha et al., 2000; Okamoto et al., 2003).

The deduced amino acid sequence of the IκBL gene shows that it comprises ankyrin repeat sequences that are known to be involved in protein-protein interactions. These sequences are also found in the IκB family of proteins allowing them to bind and thus regulate the NFκB/Rel family of transcription factors (Price et al., 2003). These and other observations suggest that IκBL may be an atypical member of this family.

As IκBL shares homology with the IκB family it is proposed to function in a similar fashion. Its genomic location close to the MHC also points to a functional role in controlling inflammation and regulating the acute phase proteins (Miterski et al.,

2004). However, the biological function of the putative IκBL protein is mainly speculative at this stage.

1.9 Summary

Sepsis is an infection-induced syndrome with a high mortality rate and the potential to utilise the majority of an ICU's budget. Ideally, a complex immunological cascade ensures a prompt, efficient protective response to invasion by pathogens. Early research, using animal models, pointed towards an excessive, poorly regulated response that was inherently pathological through the maladaptive release of endogenous inflammatory compounds. Secondary to the failure of anti-inflammatory compounds as a therapy in sepsis, an alternative theory implicating a deficient immunological defence allowing infection to become established is gaining popularity. In particular, regulatory control of the T helper cell response to infection has been widely studied and irregularities identified within this pathway have been implicated as being causative.

By examining an immune response that results in severe sepsis and comparing this to both the response observed in healthy controls and in a group that tolerate a similar infective episode with relative impunity we hope to be able to identify factors involved in differentiating an appropriate and inappropriate immune response. Central to this is the utilisation of the technique of quantitative real time polymerase chain reaction (QRT-PCR) as a surrogate for gene expression. This provides an increase in sensitivity over older, less sensitive techniques utilising ELISA based technology. Although the optimum assessment of gene function is rightly considered to be protein expression, the relatively low levels of protein expressed *in vivo* preclude widespread use of ELISA techniques in this setting, without recourse to artificially inflating protein levels by exogenous cell stimulation techniques. Recently however, a group confirmed that previous reports demonstrating a lack of correlation between protein and mRNA levels may be explained by the inadequate sensitivity of most protein

detection assays. Using a sufficiently sensitive test, such as an isotope coded affinity tagging method, confirms that mRNA levels track protein variability closely and that mRNA quantification is an accurate representation of gene expression and may be the optimal technique in situations where protein production is limited (Fu et al., 2007).

An accurate description of the pathogenic immune response in sepsis introduces the possibility of uncovering a biomarker useful for prognostication in sepsis and is the first step towards the introduction of novel targeted immunomodulating compounds.

Chapter 2 - Materials and methods

2.1 General research plan

2.1.1 Study design

We designed a prospective observational study enrolling three patient groups with the primary intention of comparing and contrasting the differing immunological responses to infectious insults of varying severity. We were primarily interested in comparing a deficient immunological response resulting in organ failure against a response that is adequately bactericidal without causing compromise to host organ function. It is this comparison that may ultimately provide us with the most valuable information regarding an adequate immunological response to an infectious insult.

2.1.2 Patients

Three groups were enrolled in this study. Ethics committee approval was received from the institutional ethics committee and informed written consent was obtained from each patient or from a relative. Two patient groups were recruited from St James's Hospital, Dublin, Ireland, which is a tertiary care university hospital. Control samples were obtained from healthy hospital and laboratory staff.

2.1.3 ICU group

A total of 62 consecutive patients with severe sepsis or septic shock, as defined by the American College of Chest Physicians / Society of Critical Care Medicine Consensus Conference (1992) were enrolled over the course of 12 months. The criteria used for the identification of severe sepsis and septic shock have been outlined in the chapter 1.

Severity of illness was measured in terms of organ impairment using a composite of global organ dysfunction. This was standardised by utilising the Simplified Acute Physiology Score (SAPS2) (Le Gall et al., 1993), Multiple Organ Dysfunction Score (MODS) (Marshall et al., 1995) and the Sequential Organ Failure Assessment (SOFA) (Vincent et al., 1996) scoring system on admission to ICU and again assessed on day 7 with the MODS and SOFA scores. The data necessary for computation of these scores are presented in tables 2.1, 2.2 and 2.3.

Early scoring systems simply counted the number of failing organs. However, the degree of dysfunction is an important variable and is a vital component of the more contemporary scores that we have used here. The SAPS2 scoring system measures severity of illness using data collected during the first 24 hours after admission to ICU whereas the SOFA and MODS score can be utilised at any point during a patient's illness. The SOFA score represents an improvement in design in comparison to the MODS score primarily in the computation of cardiovascular failure. There is a clear correlation between organ failure scores and mortality in various studies (Vincent, 2006).

Individual cardiovascular, haematological, renal, metabolic and arterial blood oxygenation variables were collected and recorded on day 1 and day 7 of ICU stay. The recorded variables represented the most significant derangements from normal values recorded by the nursing staff over each 24-hour period.

The duration of dialysis, inotrope dependence, ventilation and ICU stay was recorded for each patient.

Table 2.1: Data necessary for the calculation of the SAPS2 score

Type of admission		**Chronic diseases**		**Glasgow Coma Score**	
Medical	6	None	0	14-15	0
Scheduled surgical	0	Haematological		11-13	5
Unscheduled surgical	8	malignancy	10	9-10	7
		Metastatic carcinoma	9	6-8	13
		AIDS	17	<6	26
Age		**Systolic Blood Pressure**		**Heart Rate**	
<40	0	<70	13	<40	11
40-59	7	70-99	5	40-69	2
60-69	12	100-199	0	70-119	0
70-74	15	>=200	2	120-159	4
75-79	16			>=160	7
>=80	18				
Temperature		**If ventilated or receiving CPAP; Pa02/FiO2 ratio**		**Urine Output**	
<39°C	0	<100	11	<0.5L/24hours	11
>39°C	3	100-199	9	0.5-0.999L/24hours	4
		>=200	6	>=1L/24hours	0
Serum Urea		**White Cell Count**		**Potassium**	
<10mg/dL	0	<1000/mm^3	12	<3mEq/L	3
10-29.9mg/dL	6	1000-19000/mm^3	0	3-4.9mEq/L	0
>=30mg/dL	10	>=20000/mm^3	3	>=5mEq/L	3
Sodium		**Bicarbonate**		**Bilirubin**	
>=145mEq/L	1	<15mEq/L	6	<20mg/dL	0
125-144mEq/L	0	15-19mEq/L	3	21-150mg/dL	4
<125mEq/L	5	>=20mEq/L	0	>151mg/dL	6

For the calculation of the SAPS2 score data is collected during the patients first 24 hours after ICU admission and the following guidelines adhered to. Age; the patients age in years at their last birthday. Heart rate; the worst value in 24 hours, either low or high. Systolic blood pressure; the worst value in 24 hours, either low or high. Temperature; the highest recorded temperature in °C. PaO2/FiO2 (the ratio of arterial partial pressure of oxygen (in mmHg) to fractional concentration of inspired oxygen); to be measured when mechanical ventilation was necessary and the lowest value of the ratio used. Urinary output; if the patient spends less than 24 hours in the ICU then calculate the probable amount produced over 24 hours. Serum urea; use the highest value recorded. White cell count; use the worst (high or low) value. Potassium; use the worst (high or low) value. Sodium; use the worst (high or low) value. Bicarbonate; use the lowest value. Bilirubin; use the highest value. Glasgow coma score; use the lowest value. If the patient is sedated, record the estimated Glasgow coma score before sedation. AIDS; yes if HIV positive with clinical complications such as pneumocystis carinii pneumonia, Kaposi's sarcoma, lymphoma, tuberculosis or toxoplasma infection. Heamatological malignancy; yes if lymphoma, acute leukaemia or multiple myeloma. Metastatic cancer; yes if proven metastasis by surgery, CT scan or any other radiological method.

Table 2.2: Data necessary for the calculation of the SOFA score

PaO2/FiO2 ratio (mmHg)	**Platelet count**	**Serum bilirubin (mg/dL)**
>400 0 301-400 1 201-300 2 101-200 and MV 3 <=100 and MV 4	>150 0 101-150 1 51-100 2 21-50 3 <=20 4	<20 0 20-32 1 33-101 2 102-204 3 >204 4
Cardiovascular MAP<70 1 Dopamine<=5µg/kg/min or dobutamine 2 Dopamine >5µg/kg/min Or adrenaline<0.1µg/kg/min 3 Or noradrenaline<0.1µg/kg/min dopamine>15µg/kg/min Or adrenaline>0.1µg/kg/min 4 Or noradrenaline>0.1µg/kg/min	**Glasgow coma score** 15 0 13-14 1 10-12 2 6-9 3 <6 4	**Serum creatinine (mg/dL)** <110 0 110-170 1 171-299 2 300-440 or <500ml urine/day 3 >440 or <200ml urine/day 4

The SOFA has a maximum score of 24 and is calculated using the worst recorded variables over each 24-hour period. PO2/FiO2 ratios were not restricted to ventilated individuals. It differs from the MODS score primarily in its' calculation of the cardiovascular variable. The SOFA score uses dose and potency of inotropic medication required to maintain a normal blood pressure to characterise cardiovascular abnormality.

Table 2.3: Data necessary for the calculation of the MODS score

Pa02/FiO2 ratio (mmHg)		**Serum creatinine(mg/dL)**		**Serum bilirubin(mg/dL)**	
>300	0	<=100	0	<=20	0
226-300	1	101-200	1	21-60	1
151-225	2	201-350	2	61-120	2
76-150	3	351-500	3	121-240	3
<=75	4	>500	4	>240	4
Platelet count		**Glasgow coma score**		**Pressure adjusted heart rate**	
>120	0	15	0	<=10	0
81-120	1	13-14	1	10.1-15.0	1
51-80	2	10-12	2	15.1-20.0	2
21-50	3	7-9	3	20.1-30.0	3
<=20	4	<=6	4	>30.0	4

The MODS has a maximum score of 24 and is calculated using the worst recorded variables over each 24-hour period. PO2/FiO2 ratios were not restricted to ventilated individuals. Pressure adjusted heart rate was calculated using the following formula:
Pressure adjusted heart rate= (Heart rate X Right atrial pressure)/Mean arterial pressure.

The source of the infection necessitating the ICU admission, and subsequent infections over the course of the ICU stay, were noted. The pathogenic organisms were also recorded.

The diagnosis of respiratory tract infection (RTI) was based on chest X-ray appearance in combination with hypoxemia, symptoms, clinical signs or purulent tracheal secretions. The diagnosis of RTI was also accepted in the presence of a microorganism in pleural effusions.

Primary septicaemia was diagnosed by the presence of a positive blood culture (excluding coagulase-negative staphylococci) without an identified primary source of infection. Secondary septicaemia was defined as a positive blood culture with the recovery of an identical microorganism from a culture of the suspected primary source of infection.

Intra-abdominal infection was diagnosed with the presence of pus in the abdominal cavity verified by laprotomy, percutaneous drainage or radiological imaging.

Urinary tract infection (UTI) was diagnosed in the presence of an appropriate clinical setting or with microbiological confirmation.

The diagnosis of Central Nervous System (CNS) infection was made based on clinical signs, CT scan or positive microbiological culture from a sample of cerebrospinal fluid.

Central line infection was diagnosed by a positive microbiological culture in the presence of an appropriate clinical picture.

Skin and soft tissue infections were diagnosed by a positive microbiological culture from the suspected site or from blood cultures in the presence of appropriate clinical signs.

Mediastinal infection was diagnosed in the presence of an appropriate history and clinical signs in combination with suggestive chest X ray changes or a positive microbiological isolate.

The presence or absence of infection was verified, on a daily basis, by the ICU physician based on these criteria. Microbiological isolates were designated as commensals, contaminants or pathogens in conjunction with the microbiology team.

ICU death or survival to ICU discharge was recorded and all patients received similar standardised care.

2.1.4 Bacteraemic group

Ten bacteraemic patients were enrolled in the study from hospital wards. The clinical microbiology department identified patients on the isolation of gram-negative

bacteria from blood cultures. These patients were deemed suitable for enrolment if they had no evidence of end organ impairment or impending septic crises or critical illness. Whilst the presence of SIRS criteria did not exclude these patients, those fulfilling the criteria for severe sepsis or septic shock were excluded. We chose not to include those with gram-positive or fungal isolates, as these samples were more likely to represent contaminants and also to maintain a homogenous group of patients.

Severity of illness using the SOFA score was measured at the time of blood sampling. Demographic details, sites of infection and organisms isolated were also recorded. Patients were followed up to ensure that they were not admitted to the ICU during their hospital stay and that they were eventually discharged from hospital free from their acute illness.

2.1.5 Control group

Thirteen hospital and university staff members with no known acute or chronic illness were enrolled as a control group.

2.1.6 Blood sampling

In the ICU group blood sampling was carried out within the first 24 hours of ICU admission and again 7 days later through an indwelling central venous line or by venipuncture. In the bacteraemic group, blood was collected by venipuncture within 24 hours of the positive blood culture. Controls donated blood samples by venipuncture at one time point.

20mls of blood were collected at each donation. 10mls of whole blood was collected in EDTA anticoagulated containers for mononuclear cell purification. 5mls of whole blood was collected in EDTA anticoagulated containers for subsequent DNA analysis. 5mls of whole blood was collected in containers containing a clot activator for subsequent isolation of serum.

2.1.7 Exclusion Criteria

Exclusion criteria included:

- infection with the human immunodeficiency virus;
- patients receiving chemotherapy in the past 6 months;
- patients receiving treatment with corticosteroids equivalent to prednisolone >20mg/day for more than 14 days;
- patients on cyclosporine, cyclophosphamide or azathioprine;
- non - Irish Caucasian ethnic background;
- age less than 16 years old

2.1.8 Sample size calculation and power calculations

Prospective sample size calculation in this setting was complicated by the lack of previously published material on mRNA copy numbers in this patient cohort. Indeed most previously published studies in human populations use a technique of relative quantification of mRNA thus obviating the need to directly quantify mRNA copy numbers. As such we did not know prior to commencement of the study the standard deviation of the variables or the clinical relevance of differing copy numbers.

A number of different comparisons were made in this study but of primary interest was the difference between the bacteraemic and ICU groups in terms of mRNA levels, as this potentially allows us to predict the severity of response to an infectious insult. We anticipated that the two groups would be of different size as severe sepsis requiring ICU admission is a more common diagnosis than gram-negative bacteraemia with no organ failure in our institution.

In order to quantify the sample size we need to specify the following quantities.

- standard deviation of the variable (s)
- clinically relevant difference (δ)
- significance level (α - two-sided)
- power (1-β)

The standardised difference is then calculated as the ratio of the difference of interest to the standard deviation (δ/s). Given the problems with the quantification of the standardised difference in this case, as outlined above, we specified that we would like to be able to detect a difference between the groups of one standard deviation. The standardised difference is therefore 1.0. Using a nomogram (Whitley and Ball, 2002) (see appendix, A.1) and specifying a value of 0.2 for β, 0.05 for α and a

standardised difference of 1 we obtain a value of 30 for N, which is the total sample size if we had equal numbers in each group.

We then calculate the modified sample size (Nm). If k is the ratio of the sample sizes in the two groups (n^1/n^2) then the required total sample size is

$$Nm = N(1+k)^2/4k$$

and the two sample sizes are Nm/(1+k) and kNm/(1+k). In this study we expected to enrol approximately five times the number of ICU patients compared to bacteraemic patients. Using the above calculations, for a power of 80% at a significance level of 5% we needed to enrol at least 43 ICU patients and 9 bacteraemic patients.

As we actually enrolled 62 ICU patients and 13 bacteraemic patients we can calculate the power of the study using the same nomogram. As we have unequal sample sizes we calculate an effective sample size (N).

$$N = 4Nmk/(1+k)^2, \text{ where } k=n^1/n^2 \text{ and } Nm=n^1+n^2$$

Using this formula to derive N we can draw a straight line between N and a standardised ratio of 1 and reading from the nomogram we conclude that the power in this study is close to 90% at an α level of 5%.

2.1.9 Ethical issues

It is possible that research which utilises biomarkers and genetic information may have an impact on both the patient and their family in the future. These biomarkers have the potential to provide important information about the biological effect of certain exposures and the susceptibility to disease for that individual and their family. No medical interpretation of individual results of the research was provided to study volunteers or to family, whilst also recognising that study subjects have rights to appropriate information before, during and after the study. All reasonable efforts were made to ensure that stored genetic and biological material could not be traced back to the donor. Confidentiality of the data was ensured and access was limited to directly concerned researchers only. The data were stored and reported in a manner that did not identify study participants to a third party.

Ethical approval was obtained from The Federated Dublin Voluntary Hospitals and St. James's Hospital Joint Research Ethics Committee at the start of the study. The Committee operates according to the general principles of medical ethics including the Declaration of Helsinki. The Committee also complies with the relevant provisions of the International Conference on Harmonisation Guidelines on Good Clinical Practice and with Clinical Trials legislation enacted in Ireland. Care was taken to inform the participants of their freedom to refuse participation in the study. An information leaflet was provided to each participant or a consenting family member.

2.2 DNA standards

2.2.1 Pre-prepared cytokine standards

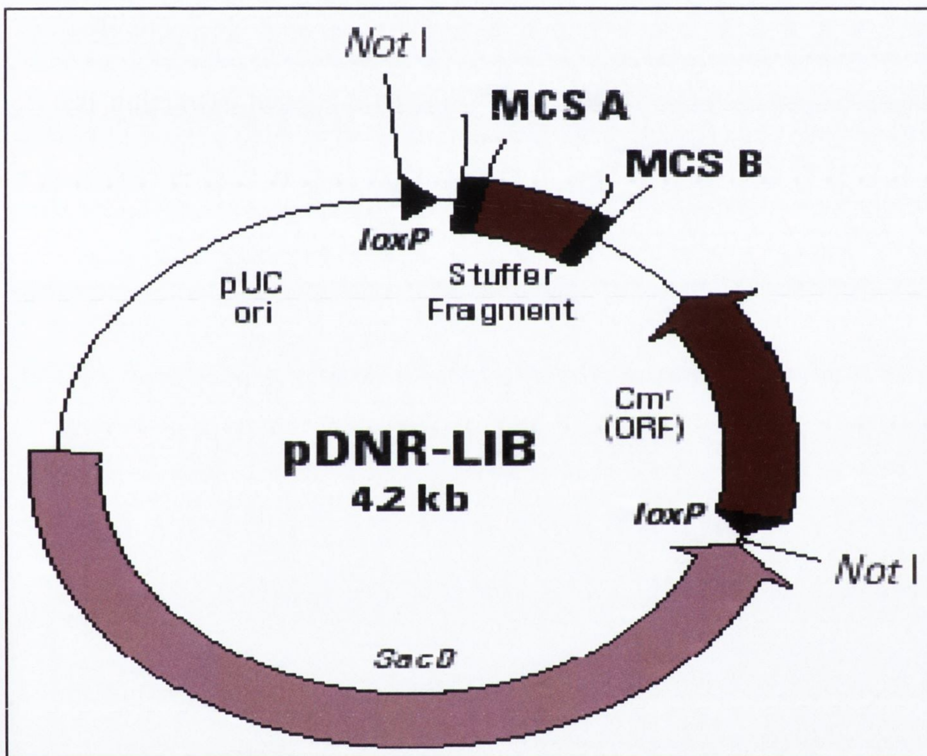
The DNA standards for TNFα, IL-10, β-actin, IL-12p35, IFNγ, IL-18, IL-4 and IL-23p19 consisted of a cloned PCR product that encompassed the quantified amplicon. This was prepared by PCR from a cDNA population containing the target mRNA. These standards were a kind gift from Patrick Stordeur, Hopital Erasme, Brussels, Belgium. Primer sequences and reaction conditions are given in the appendix (A.2).

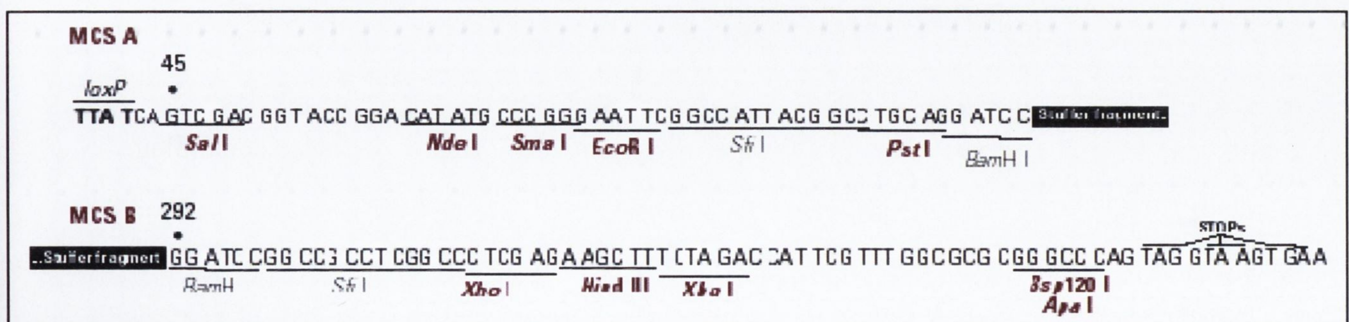
Stock solutions of standards, containing 10^9 (IFNγ, B-actin, TNFα, IL-23p35 and IL-4) or 10^{10} (IL-12p35 and IL-10) copy numbers per μl, were aliquoted and stored at -20°C. A dilution series from 10^9 to 10^2 copy numbers per μl was prepared in each case and stored at 4°C. The standards were diluted in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) containing double-stranded herring DNA (Sigma) at 10μg/ml.

2.2.2 Preparation of the IL27p28 standard

A vector carrying the IL27p28 coding sequence was purchased from Open Biosystems. It consisted of a 1.9kb cDNA clone inserted into a 4.2 kb vector encoding a chloramphenicol resistance marker. The vector is designated pDNR-LIB (figure 2.1).

Figure 2.1



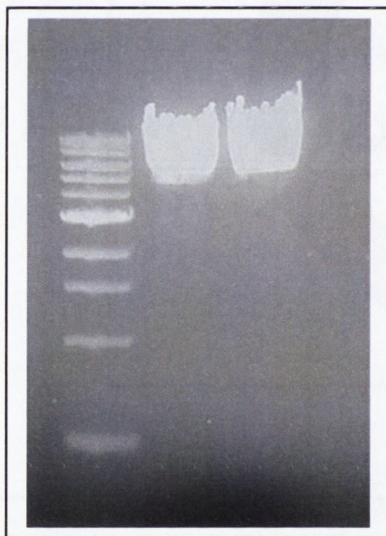


Map of the pDNR-LIB vector. MCS, multiple cloning site. The stuffer fragment is replaced by the IL27 cDNA insert. Unique restriction sites are shown in bold or in colour.

Following transformation, a chloramphenicol resistant colony was streaked onto a chloramphenicol (30μg/ml) LB agar plate and incubated at 37°C overnight. A single colony was isolated from this plate and streaked onto another plate. A well-isolated colony from this second plate was then used to inoculate a liquid culture grown overnight at 37°C. These steps ensure the isolation of a clone of a single bacterium.

Small and large-scale plasmid DNA isolation was performed from the liquid culture using Qiagen mini or midi kits (Qiagen GmBH, Germany) respectively, following the manufacturers instructions. Plasmid DNA from a typical extraction is shown in figure 2.2.

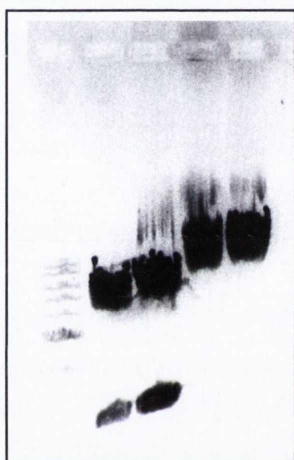
Figure 2.2



DNA gel. This gel contains, from left to right, a 1kb ladder and 2 lanes containing supercoiled plasmid DNA obtained from the midi-kit.

Single and double restriction digestion procedures were performed by the methods of Sambrook et al (1989, Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York) using EcoR1 (single digestion) and Xho1 and visualised on a gel as in figure 2.3.

Figure 2.3



DNA gel. From left to right this gel contains a 1kb ladder, two lanes containing double digested plasmid DNA and two lanes containing plasmid DNA having undergone a single digestion.

2.2.3 DNA Gels

DNA was separated on 1% agarose gels containing Ethidium bromide 0.5μg/ml and Tris-Borate-EDTA (TBE) buffer (Sigma). The average run was at a voltage of 135V for 35 minutes. Ethidium bromide-stained DNA was visualised on an ultraviolet light box and captured using a Kodak, 440CF documentation system.

2.2.4 Gel purification

The IL-27 fragment was extracted from the agarose gel using a QIAquick Gel Extraction Kit (Qiagen) following the manufacturers instructions. The band of interest was carefully removed from the gel using a scalpel. This was then placed in a spin-column that contains a DNA binding silica gel. DNA adsorbs to this in the presence of high salt buffer while contaminants pass through the column as impurities are washed away. Tris elution buffer is added to the column and left to stand for 1 minute before the DNA was eluted with the elution buffer.

The plasmid DNA was then quantified using the Nanodrop, as described in a later section, giving a concentration usually in μg/μL (see page 63 and the appendix (A3)).

2.2.5 Determining the volume of plasmid DNA corresponding to copy numbers of target nucleic acid sequences

First, it is necessary to calculate the mass of a single plasmid molecule. The plasmid size value is inseted into the formula below:

$m = (n)(1.096 \times 10^{-21}$ g/bp); where m=mass and n= plasmid size (bp), where the size of the entire plasmid (plasmid + insert) is used in this calculation. Using as an example IL-27:

$m = 5277$ bp(1.096×10^{-21}) g/bp

$= 5.78 \times 10^{-18}$ g = mass of 1 plasmid molecule

We then calculate the mass of plasmid containing the copy numbers of interest, in this example 10^{11} copies:

10^{11} copies $\times 5.78 \times 10^{-18}$ g/ copy =

5.78×10^{-7} g

The concentrations of plasmid DNA needed to achieve the copy numbers of interest were calculated by dividing the mass needed by the volume to be pipetted into each reaction. For this example, 5µL of plasmid DNA solution is pipetted into each PCR reaction. For 10^{11} copies;

5.78×10^{-7} g / 5µL = 1.16×10^{-7} g/µL.

We then prepare a serial dilution of the plasmid DNA. The following formula is used to calculate the volume needed to prepare the 10^{11} copy standard dilution. For this example, the stock solution of plasmid DNA was taken as having a concentration 1.5µg/µL as determined by spectrophotometric analysis using the nanodrop.

$C_1V_1 = C_2V_2$

$(1.5\mu g/\mu L)(V_1) = (1.16 \times 10^{-7}\ g/\mu L)(100\mu L)$

$V_1 = 7.73\mu L$

Therefore, for 10^{11} copy numbers of IL-27 / µL add 7.73 µL plasmid DNA to 92.27µL of dilutent and from this point prepare a serial dilution of the plasmid DNA.

2.2.6 Preparation of the IKBL standard

Human IKBL cDNA was purchased from Invitrogen (H-X77909-M Invitrogen clone). The complete coding sequence was amplified using the following primers that contained introduced restriction sites Nde1 and Xho-1:

- Forward primer: ACTCAGATCATATGAGTAACCCCTC
- Reverse Primer: TGGATCCTCGAGTCACGGTACCTTGAG

The reaction conditions for the PCR were 95°C for 15 min; 95°C for 30 s; 58°C for 1 min; 72°C for 1 min for 35 cycles.

The amplified products were gel purified as described, confirmed by sequencing (Dept. of Biochemistry, Cambridge University, UK) and quantified using nanodrop technology as described below. This PCR product was then cloned into a GFP containing vector. This vector was subsequently transformed into *E. Coli* and purified by streaking on kanamycin (30μg/ml) containing LB agar plates.

Isolation and quantification then proceeded as previously described. For calculation of the volume of plasmid DNA needed for specific copy numbers we took the size of the vector 4.7 kb and the size of the insert as 1155 base pairs and performed the calculations as before.

2.3 Isolation of cDNA from whole blood

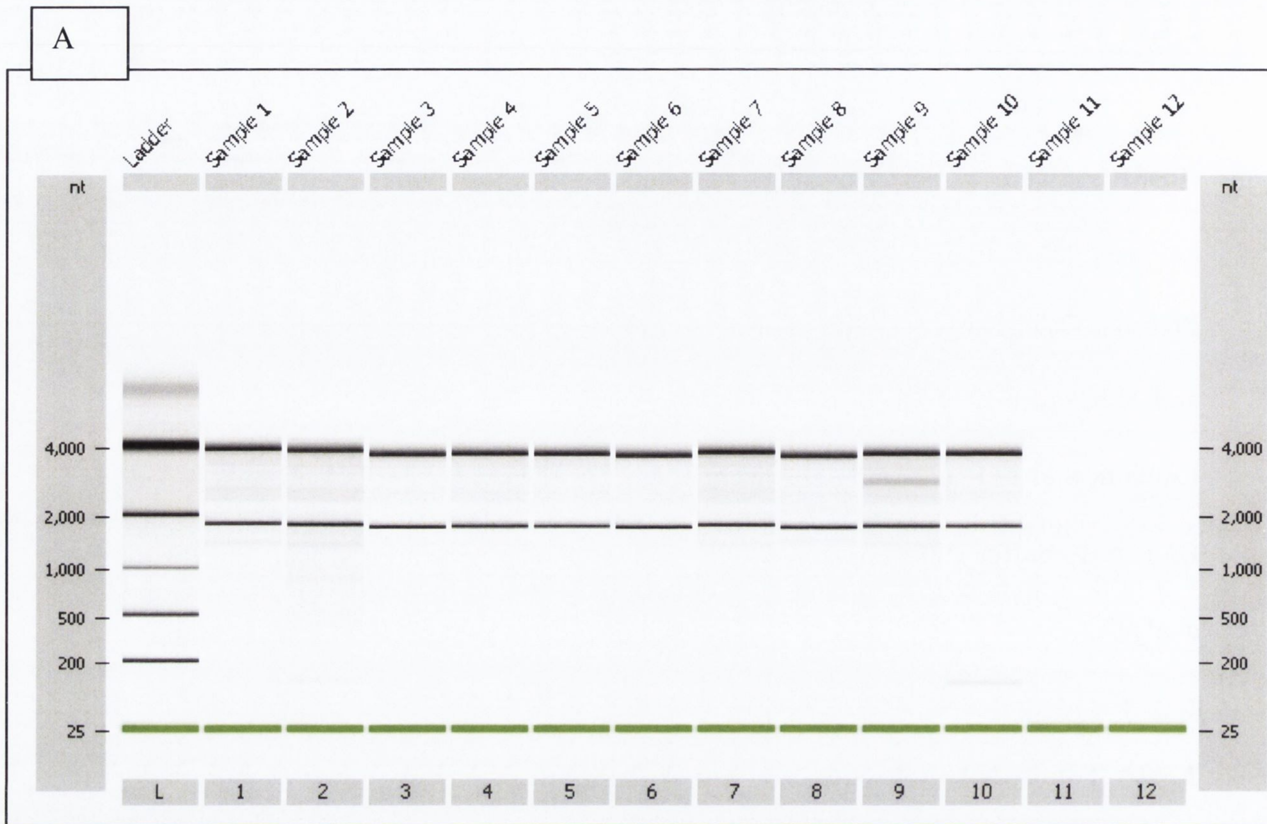
2.3.1 Mononuclear cell isolation

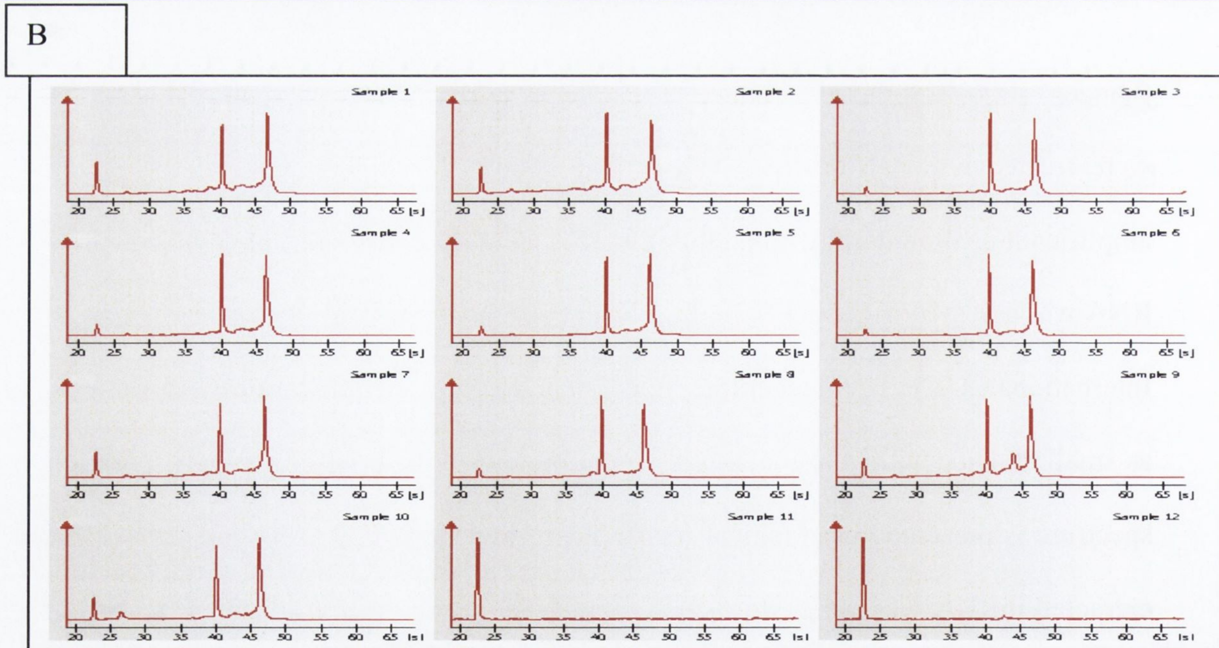
Immediately on obtaining a blood sample, peripheral blood mononuclear cells (PBMCs) were purified by density gradient centrifugation of 10mls of EDTA anticoagulated blood, at 1200rpm for 30 minutes, over lymphoprep (Nycomed Pharma, Oslo, Norway). The interface buffy layer of cells was collected by pipetting and subsequently washed for 4 cycles in Hanks Balanced Salt Solution without Calcium and Magnesium. Cells were subsequently stored at -80°C, resuspended in RLT lysis buffer (Qiagen) and 6μl 14.3M 2-Mercaptoethanol (Sigma) until further analysis.

2.3.2 Total RNA extraction and quantification

Total RNA was isolated from lysed PBMC using a commercially available kit (Qiagen) following the manufacturers instructions. During this procedure all samples were treated with RNase-free DNase (Qiagen) for 15 minutes, in order to avoid amplification of contaminating genomic DNA. The quantity and purity of extracted RNA was assessed using the Nanodrop ND 1000 UV-Vis spectrophotometer (Labtech International, UK). This technology allows nucleic acid quantification from samples as small as 1 μL with consequent savings in biological material. A typical absorption spectrum is presented in graphical format in the appendix (A.3). The integrity of the extracted mRNA was verified on an Agilent 2100 Bioanalyser using the RNA Nano LabChip kit (Agilent, CA, USA) (figure 2.4). Whereas standard agarose RNA gels require up to 200ng of RNA, this method requires just 10ng of RNA thus offering significant advantages when the amount of starting material is limited.

Figure 2.4





Agilent 2100 Bioanalyser. A) The two bands depicted in the gel-like image are the 28s (top) and 18s (bottom) fragments of ribosomal RNA. The bands are sharp and clear with the 28s band displaying approximately twice the intensity of the 18s band indicating that the RNA has not been degraded.
B) The electropherograms display high quality total RNA with the 18s and 28s fragments clearly visible at 40 seconds and 47 seconds.

2.3.3 Reverse Transcription

Preparation for reverse transcription took place in a laboratory area separate from that used for dealing with DNA and QRT-PCR. One set of pipettes, reagents and disposable pipette tips were used exclusively for this step to prevent contamination of the samples.

Total RNA was reverse transcribed as follows: 11.15µl of water containing 500ng of total RNA was mixed with 2µl 100µM Random Primers (Invitrogen, CA, USA) and incubated at 65°C for 10 minutes to denature any RNA secondary structure present. 16.85µl of the reverse transcription mix containing the following components were then added: (1) 3µl 0.1M DTT; (2) 4.5µl Dimethyl Sulfoxide: (3); (4) 1.25µl Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Invitrogen); (5) 6µl 5XFirst Strand Buffer (Invitrogen); (6) 1.5µl 4mM dNTPs (Promega, Madison MI, USA); 0.6µl RNasin (Promega) 10u/µl. The samples were then incubated at 37°C for 1 hour.

Although the reverse transcription step can be primed using random hexamers, specific primers or oligo-dT primers we chose random hexamers to ensure maximisation of potentially small amounts of available mRNA.

We used MMLV reverse transcriptase, due to its lack of inherent RNAse-H activity. This is important when synthesising lengthy amplicons during PCR.

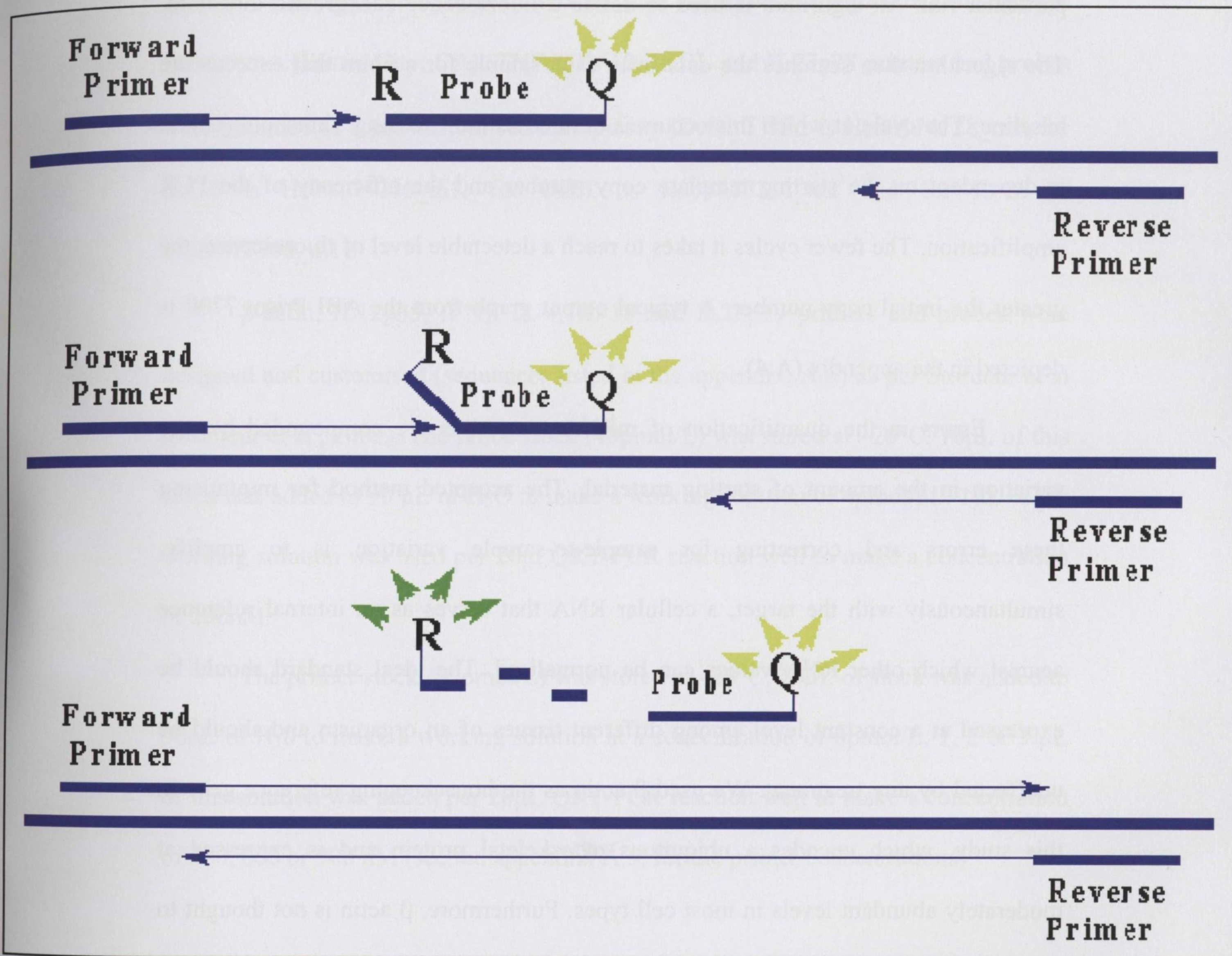
2.4 Quantitative Real-Time Polymerase Chain Reaction

2.4.1 Theory

QRT-PCR is a technique used to determine the number of copies of the RNA/ cDNA DNA segment of interest present in a sample. While the procedure follows the general pattern of standard PCR, the DNA is re-quantified after each round of amplification; hence in real time. The TaqMan assay is based on detecting a specific PCR product. Amplification proceeds using sequence specific primers and Taq DNA polymerase. Detection results from hybridisation of the target sequence to a doubly labelled flurogenic probe. In an intact TaqMan probe, the fluorescent dye and the quencher engage in fluorescence resonance energy transfer (FRET) and thus the probe is non-fluorescent. During PCR, the probe is cleaved by the inherent 5'-deoxynuclease activity of the Taq DNA polymerase. This only occurs when the TaqMan probe hybridises to the target DNA that is being amplified. As a result, the fluorophore is cleaved from the probe and diffuses away from the quencher allowing a fluorescence signal to be generated. This is demonstrated in figure 2.5.

The ABI Prism 7700 contains a built-in thermal cycler with 96-well positions, and is able to detect fluorescence between 500nm and 660nm. Fluorescence is induced during the RT-PCR by distributing laser light to all 96 samples contained in thin-walled reaction tubes via a multiplexed array of optical fibres. The resulting fluorescent emission returns via the same fibres.

Figure 2.5



The principals of QRT-PCR. PCR proceeds using sequence specific primers while a sequence specific probe binds downstream of the primer. The 5'-deoxynuclease activity of the Taq DNA polymerase cleaves the probe and the reporter dye is separated from the quencher resulting in fluorescence. The Taq DNA polymerase then finishes amplifying the area of interest.

The ABI prism establishes the levels of background fluorescence for each particular run. An algorithm is used to define a fluorescence background threshold. The algorithm then searches the data from each sample for a point that exceeds the baseline. The cycle at which this occurs is defined as the Crossing Threshold (Ct). It is dependent on the starting template copy number and the efficiency of the PCR amplification. The fewer cycles it takes to reach a detectable level of fluorescence, the greater the initial copy number. A typical output graph from the ABI Prism 7700 is depicted in the appendix (A.4).

Errors in the quantification of mRNA transcripts are compounded by any variation in the amount of starting material. The accepted method for minimising these errors and correcting for sample-to-sample variation is to amplify, simultaneously with the target, a cellular RNA that serves as an internal reference against which other RNA values can be normalised. The ideal standard should be expressed at a constant level among different tissues of an organism and should be unaffected by any treatment. We used β actin as the housekeeping reference gene in this study, which encodes a ubiquitous cytoskeletal protein and is expressed at moderately abundant levels in most cell types. Furthermore, β actin is not thought to be affected by any of the standard ICU medications.

Following normalisation using a housekeeping gene, absolute quantification then requires the construction of an absolute standard curve for each individual amplicon using serial dilutions of target DNA of known concentrations. This allows precise determination of copy numbers of the gene of interest by comparison with the standard curve.

2.4.2 Primers and probes

All primers and probes used in this study were synthesized at Applied Biosystems (Foster City, CA). TNFα, IL-10, IKBL and IL27p28 primers and probes were obtained as a pre-customised mix (Assay ID for TNFα is Hs00174128_m1, for IL-10 is Hs00174086_m1, for IKBL is Hs00428211_m1 and for IL-27 is Hs00377366_m1).

β-actin, IL12p35, IFNγ, IL-4, IL-18 and IL23p19 primers and probes were designed and customised (sequences listed in the appendix, A.5) as per Stordeur et al (Stordeur et al., 2002). The probe stock (40pmol/L) was stored at –20°C. 10μL of this stock was added to 90 μL of H_2O to make a working solution of 4pmol/μL. 1μL of the working solution was used per 20μl QRT-PCR reaction well to make a concentration of 200nM.

The primer stock (60pmol/L) was stored at –20°C. 10μL of stock was added to 90μL of H_20 to make a working solution at a concentration of 6pmol/L. 1, 2 or 3 μL of this solution was added per 20μL QRT-PCR reaction well to make a concentration of 300, 600 or 900 nM (see the appendix, A.5, for the primer concentrations).

2.4.3 Setting up the QRT-PCR reaction plate

The plate is set up in an extractor hood in a dedicated area not used for DNA work. A separate set of pipettes, reagents and disposable pipette tips are used exclusively for the QRT-PCR step to avoid contamination.

The PCR reactions were carried out in an ABI Prism GeneAmp 7000 Sequence Detection System (Applied Biosystems). All reactions were performed either in triplicate or in duplicate.

Each QRT-PCR plate contained:

1) A dilution series of the DNA standard of the gene being quantified (in concentrations ranging from 10^6 to 10^2 copies per μL),

2) A dilution series of the β actin DNA standard (in concentrations ranging from 10^9 to 10^5 copies per μL),

3) Triplicate or duplicate wells containing patient cDNA and primers and probe for the gene being quantified,

4) For each patient being analysed on the plate, β actin is quantified concurrently for that patient,

5) non-template controls (NTCs) containing water in place of the patient sample are run on each plate.

The concentrations of the dilution series are designed to encompass the expected range of copy numbers of the genes being examined. Lack of product in the NTC wells confirms that no contaminanting DNA is present in the reaction mix. A sample 96 well plate set-up is depicted in the appendix (A.6).

Primers, probes, cDNA and DNA standards were thawed on ice whilst preparing the PCR mix. Thermocycling was carried out in a 20μl final volume containing:

- water up to 20μl;
- 10μl Mastermix (Applied Biosystems) containing ampli-Taq Gold® DNA polymerase and proprietry buffer designed to increase the performance and reliability of the 5' nuclease activity.
- 1, 2 or 3 μl of 6 pmol/μl forward and reverse primers (final concentration 300,600 or 900nM, see appendix, A.5);
- 1μl of 4 pmol/μl Taqman Probe (final concentration 200nM) or 1μl of pre-customised primer/ probe mix with default primer and probe concentrations (appendix, A.5);
- 0.8μl of DNA standard dilution or 2.4μl patient cDNA.

The 96-well optical reaction plate (Applied Biosystems) was centrifuged briefly and covered with an optical adhesive cover prior to analysis.

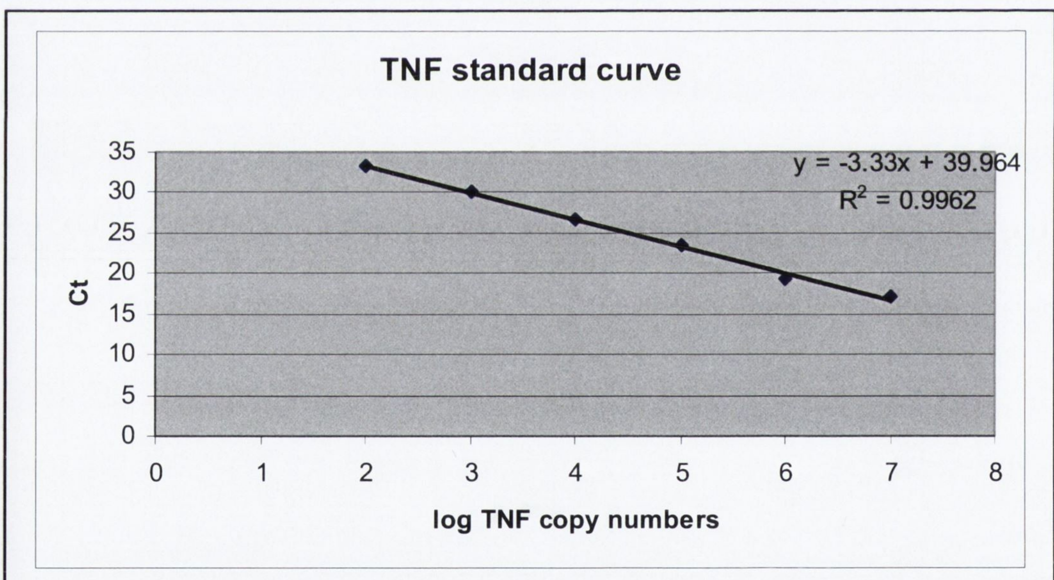
After an initial denaturation step at 95°C for 10 minutes, temperature cycling was initiated. Each cycle consisted of 95°C for 15 seconds and 60°C for 60 seconds, the fluorescence being read at the end of this second step. In total, 40 cycles were performed.

2.4.4 Interpreting the results

In order to quantify gene expression a standard curve was constructed for each QRT-PCR run, for each selected gene from serial dilutions of the relevant standard. This was constructed by plotting log standard copy numbers against Ct values obtained from QRT-PCR (figure 2.6).

All calibration curves showed correlation coefficients >0.99, indicating a precise log-linear relationship.

Figure 2.6



Standard curve for quantification of copy numbers. Log standard copy numbers are plotted on the X axis and Ct values obtained during QRT-PCR along the Y axis. The equation is the equation for the straight line along with an r^2 value.

The efficiency (E) of the PCR reaction was then estimated from each standard curve using the slope of the line. Using the example in figure 2.6 and the formula:

$E=((10^{-1/\text{slope of the standard curve}})-1) \times 100;$

E was found to be 99.53%. This indicates that the PCR amplicon approximately doubles in quantity during the geometric phase of its PCR amplification.

The mean efficiency of the standard curves for all target genes was found to be 98.75% +/- 4.4%.

The mRNA copy numbers were then calculated for each patient sample by using the relevant standard curve to convert the obtained Ct value into mRNA copy numbers. Results were then expressed in absolute copy numbers after normalisation against β-actin mRNA (mRNA copy numbers of cytokine mRNA per 10 million β-actin mRNA copy numbers).

2.5 Serum

2.5.1 Serum collection

Serum was obtained from whole blood clotted for 30 minutes at room temperature and spun at 2500rpm for 10 minutes. The resulting serum was stored at -80°C until further analysis.

2.5.2 Cytokine Enzyme Linked Immunosorbant Assay (ELISA)

We first confirmed the presence of protein in the serum samples using the Bradford method (Bradford, 1976), in a random selection of samples prior to ELISA measurement. This is a colorimetric assay for measuring total protein concentration and involves the binding of Coomassie Brilliant Blue dye to protein. Bradford reagent (Bio-Rad Laboratories, GmbH, Munich, Germany) is mixed with a protein standard, Bovine Serum Albumin (BSA) (Sigma), in concentrations ranging from 0 to 1mg/ml). Absorbance at 595nm was measured using a microplate autoreader (Spectrafluor Plus, Tecan, Toronto, Canada). Using the standard curve generated from the BSA the unknown protein concentrations were determined.

Serum TNFα, IL-10, IL-6 and IFNγ concentrations were measured by ELISA (R+D systems, Minneapolis, USA) following the manufacturers instructions.

Capture antibodies for TNFα consisted of mouse anti-human TNFα. The detection antibodies were biotinylated goat anti-human TNFα and the standard curve was generated using serial dilutions of recombinant human TNFα. The lower limit of detection for TNFα was 15.625pg/ml.

Capture antibodies for IL-10 were mouse anti-human IL-10. The detection antibodies were biotinylated goat anti-human IL-10 and the standard curve was

generated using recombinant human IL-10. The lower limit of detection for IL-10 was 46.875pg/ml.

Similarly, capture antibodies for IL-6 and IFNγ were mouse anti-human IL-6 and anti-human IFNγ. The detection antibodies were biotinylated goat anti-human IL-6 and anti-human IFNγ and the standard curve was generated using the respective recombinant proteins. The lower limit of detection for IL-6 was 9.375pg/ml and 15.625pg/ml for IFNγ.

After addition of Streptavidine-HRP, followed by the Substrate Solution (1:1 mixture of H_2O_2 and Tetramethylbenzidine), the plates were left in the dark for 10-30 minutes before stopping the reaction with the addition of H_2SO_4. Absorbencies were read at 450 and 595nm in a microplate autoreader (Spectrofluor Plus, Tecan).

All samples were tested in duplicate.

2.5.3 ADMA quantification

ADMA was measured using a microtitre plate assay developed by Schulze et al in 2004 (Schulze et al., 2004), and manufactured by DLD Diagnostika Ltd. Hamburg , Germany. ADMA is bound to the solid phase of the microtitre plate. ADMA in the samples is then acetylated by pre-treatment with acylation buffer, (1mol/L Tris-HCl-buffer) and lyophilised acylation reagent (N- hydroxysuccinimido carbonic acid dissolved in 1.5 ml dimethyl formamide) followed by incubation for 15-20 hours at 4° C. 50μl of treated samples then compete with solid phase bound ADMA for a fixed number of rabbit anti-ADMA antiserum binding sites. After washing the antibody bound to the solid phase ADMA is detected by anti-rabbit/peroxidase. The reaction is monitored at 450nm using an automated microtitre plate reader (Bio Tex Elx800) within one hour. The amount of antibody bound to the

solid phase ADMA is inversely proportional to the ADMA concentration of the sample.

Standards are provided with the assay kit and cover the range 0.05-5.0 μmol/L ADMA. On a semi logarithmic graph the concentration of the standards (x axis, logarithmic) are plotted against their optical densities (y axis, linear). The concentration of the samples can be read directly from this standard curve by using their average optical density. The sensitivity of the assay is 0.05μmol/L.

2.6 DNA analysis

2.6.1 DNA extraction

From whole blood stored at –80°C, DNA was extracted and purified using a QIAamp DNA blood Mini Kit (Qiagen, Hilden, Germany). This kit uses spin columns and is suitable for whole blood treated with EDTA and yields up to 6μg DNA from 200μL of whole blood. Salt and pH conditions in the lysate ensure that protein and other contaminants, which can inhibit PCR and other downstream reactions, are not retained on the QIAamp membrane. DNA bound to the QIAamp column is then washed in two buffers, AW1 and AW2, which significantly improves the purity of the eluted DNA. This purified DNA is then eluted from the QIAamp spin column in water.

Concentration and purity of DNA was determined using the Nanodrop ND 1000 UV-Vis spectrophotometer as previously described (A.3). Purified DNA was then stored until further analysis at -20°C.

2.6.2 Amplifluor Assay

The TNFα, DDAH II, IKBL and IL-10 polymorphisms were genotyped commercially by Kbiosciences, UK using Amplifluor technology.

The Amplifluor (Chemicon International, Inc., CA, U.S.A.) SNP genotyping system is based on a fluorescent (energy transfer) hairpin primer. The primer has little fluorescence in the native closed state, but upon incorporation into an amplicon during PCR, the hairpin unfolds separating the fluorophore and quencher producing a signal that can be easily detected. The amplifluor primer consists of four parts:

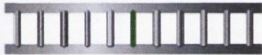
1) The hairpin structure in the SNP primer. This has been designed to provide optimal thermodynamic stability for the molecule. 2) The fluorophore (FAM for one allele specific primer and sulforhodamine (SR) for the other allele specific primer). This is attached to the 5' end of the primer at the base of the hairpin structure and is in close proximity to the quencher when the primer is in the hairpin conformation. 3) The quencher; dimethylaminoazosulfonic acid. This non-fluorescent azo dye effectively quenches a variety of fluorophores while allowing Taq polymerase to read through it. 3) The Z tail. This is composed of a unique sequence of 21 bases at the 3' end of the primer. In a single reaction for a biallelic SNP two distinct Z tails are used, one on the 3' end of the green Amplifluor and another on the 3' end of the red Amplifluor. Unlabelled, allele specific ologonucleotides are synthesized with the same green or red Z tails on their 5' end. Once the Z tail is incorporated into the amplicon, the primer is able to prime the amplification by Taq (Fig 2.7).

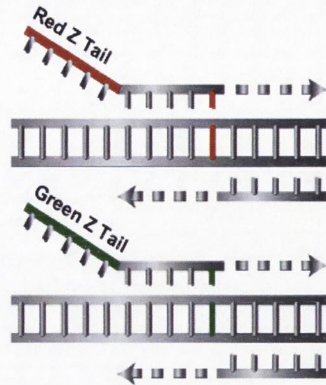
Figure 2.7

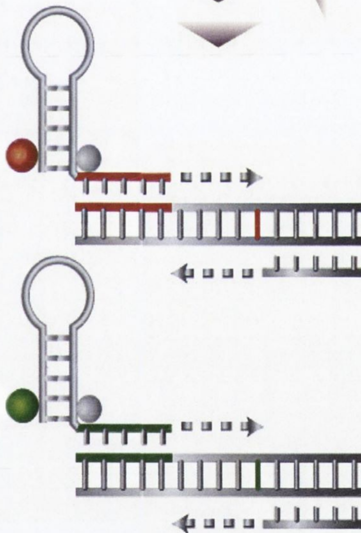
Heterozygous DNA Sample

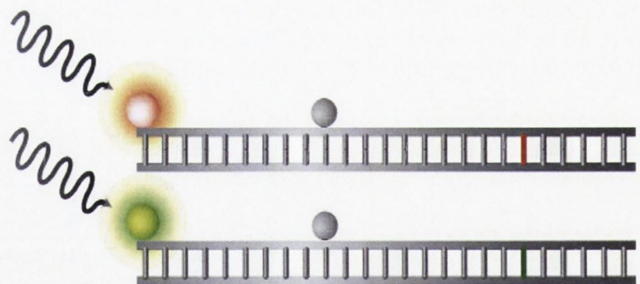
Allele 1

Allele 2









Amplifuor assay. First, the two Z-tailed, unlabeled, allele-specific primers with the common reverse primer initiate a competitive allele specific PCR reaction. During early rounds of PCR, the allele specific primer with its unique "Z" tail is incorporated in the amplicon. The corresponding amplifluor SNP primer recognizes the complement of the "Z" tail sequence and is able to prime off that sequence. Incorporation of the Amplifluor primer into an allele-specific amplicon melts its hairpin structure, thus separating the fluorophore from the quencher and generating the fluorescent signal. (Figure taken from Chemicon International manual).

2.6.3 Taqman genotyping

Taqman technology was used to genotype the PAI 4G/5G polymorphism. This method combines PCR amplification and detection in the same reaction and is based on the 5'-3' exonuclease activity of *Taq* DNA polymerase. A PCR is performed using primers that will amplify the DNA region containing the polymophism of interest. Included in the reaction are two allele-specific fluorogenic probes, each consisting of a different fluorescent reporter dye and a quencher. During PCR, the 5' nuclease activity of the *Taq* DNA polymerase digests the allele-specific probe bound to the region of the SNP, releasing the fluorescent dye from the quencher and allowing generation of a fluorescence signal. Depending on which dye signal is generated, the SNP alleles can be determined.

Each reaction contains 2ng of genomic DNA. Alleles are subsequently identified using software that provides scatter plots of allelic calls. An example of the scatterplot produced is given in the appendix (A.7).

Specific primers and fluorescent dye-labelled Taqman MGB probes were provided by Applied Biosystems (Perkin Elmer, California, USA). The primer sequences, reagent mix for the PCR reaction and PCR reaction conditions are given in the appendix (A.8). The PCR procedure was performed on a Peltier Thermal Cycler 200 (Global Medical Instrumentation, Ramsey, Minnesota, USA). Allelic discrimination was then determined using an ABI prism 7000 Sequence Detection System (Applied Biosystems / Perkin Elmer).

2.6.4 Haplotype estimation

The genotyping methods described above are able to determine which two alleles are present at each locus but are unable to provide haplotype information. Therefore, we do not know from SNP genotyping which combinations of alleles are present on each chromosome. Information regarding the haplotypes is helpful as genetic inheritance operates through the transmission of chromosomal segments, with limited numbers of common haplotypes representing those segments in most populations. Thus, the ability to identify haplotypes is a more powerful and also cost effective approach to genetic mapping, since not all genetic variants on a haplotype need be identified.

Both experimental and statistical models are available for haplotype determination with the later having the advantages of speed and cost savings. As data on related individuals was unavailable in this study to assist in determination of the haplotypes, we instead inferred haplotypes from unphased genotype data. Several statistical approaches are available when faced with this problem, including Clark's algorithm (Clark, 1990) and the maximum likelihood estimation of haplotype frequencies via the Expectation Maximisation (EM) algorithm (Excoffier and Slatkin, 1995). In this case we utilised PHASE version 2 (Stephens and Donnelly, 2003) and Haploscore software (Schaid et al., 2002) (http://www.stat.washington.edu/stephens/software.html).

PHASE uses a Bayesian statistical method for reconstructing haplotypes from population genotype data and is regarded as slightly more accurate than other methods for reconstructing haplotypes (Stephens and Donnelly, 2003). Haploscore uses the EM algorithm and has the useful attribute of implementing a permutation based statistical test of association allowing the distribution of haplotypes between groups

(i.e. between high and low cytokine producers) to be tested for homogeneity. Both approaches produced comparable estimates of haplotype frequencies.

Genepop software was used to assess if the genotype distributions were in Hardy-Weinberg equilibrium (http://wbiomed.curtin.edu.au/geneopop/). Files were then converted from Genepop format to PHASE format using the Arlequin software package.

The input file for PHASE specifies the number of individuals to be analysed, the number of loci typed, the nature and physical positions of these loci (in this case SNPs) and the genotypes for each study subject.

Output from PHASE consists of a number of different files. The first contains a summary of the individual haplotype estimates of each individual. Other files contain more results including estimates of sample haplotype frequencies, list of most likely pairs of haplotypes carried by an individual, estimates of recombination parameters across the region, and goodness of fit of the estimated haplotype to the underlying model.

Haplotypes were determined for the IL-10 promoter using SNPs located at position IL-10 –1082, IL-10 –819, IL-10 –592. The following SNPs on chromosome 6 were analysed; TNFα –238, -308, -857, - 863, DDAH II –449 and IKBL –62. PHASE was used to infer the haplotypes present in the patient population using markers available across the TNFα and IL-10 promoter.

2.7 Statistics and data presentation

All data were analysed using JMP software (SAS institute, USA). A p value of 0.05 was considered significant.

Data were assessed for conformity to a normal distribution using a Shapiro-Wilk W ' test. Paired means incorporating non-parametric data were analysed using the wilcoxin signed-rank sign test. Between group comparisons involving non-parametric continuous data were analysed using the wilcoxin rank sum test. Continuous non-paramaetric data were subsequently expressed as the median with the interquartile range (IQR) in parenthesis.

Between group comparisons for parametric continuous data was analysed using a paired t-test for matched pairs and a t-test for independent groups. Parametric data were sunsequently expressed as the mean with the standard deviation (SD) in parenthesis.

Categorical data were analysed using a chi-squared test.

Hierarchical clustering was used to identify groups of relatively homogenous observations. Ward's procedure, which generates clusters that minimse the squared Euclidean distance to the center mean, was used to organise objects into a hierarchical structure.

Chapter 3 - Results (1): Demographics and clinical variables pertaining to the patient groups

3.1 ICU group

3.1.1 Description of clinical and biochemical variables on day 1 of ICU stay

The recruitment period was 12 months during which time 62 ICU patients fulfilled the criteria for inclusion to the study, and were enrolled. The patient's median age was 70 years (IQR 55.5 to 78 years). The range of ages was from 22 to 88 years. Whilst more males (34 (54.8%)) than females (28 (45.2%)) were recruited, this difference was not statistically significant (p=0.45).

Blood samples were obtained from 52 (84%) patients on day 1. A median of 15.5 hours (IQR 10 to 20 hours) elapsed from patient admission to the ICU and the first series of blood sampling. The median interval between hospital admission and ICU admission was 2 days (IQR 0 to 13 days).

Admission to ICU was then analysed for seasonal variation. 19 (30%) patients were admitted in the spring, 11 (18%) in the summer months, 18 (29%) patients during the autumn and 14 (23%) during the winter. The difference in patient numbers being admitted per season was not found to be significant (p=0.44). Additionally, there was no association between the season of admission and mortality or severity of illness as assessed by the organ failure scores on day 1. The source of the infectious insult was not associated with any seasonal variation nor was the age of the patient associated with any seasonal variation.

On admission to ICU, 42 (67.7%) patients fulfilled the criteria for septic shock whilst the remaining 20 (32.3%) fulfilled the criteria for severe sepsis only. For the purposes of this study "inotropes" refers to the administration of either adrenaline or noradrenaline in order to maintain a mean arterial blood pressure (MAP) greater than 60mmHg. The majority of patients requiring inotropes on admission to ICU received

noradrenaline (42 patients) with a minority (3 patients) receiving both noradrenaline and adrenaline and no patients receiving adrenaline alone. In those patients receiving noradrenaline, the median highest dose administered on day 1 was 13.5mcg/min (IQR 5 to 27 mcg/min).

All subsequent data in this section refers to patient variables on admission to the ICU unless otherwise stated.

The median MAP was 65mmHg (IQR 60 to 75 mmHg) and was significantly lower in those patients requiring inotropes (75mmHg Vs 62.5mmHg, p=0.0009). Mean central venous pressure (CVP) was 12mmHg (SD 5.6mmHg) and was higher in the group receiving inotropes (9mmHg Vs 13mmHg, p=0.02).

Median pH was found to be 7.31 (IQR 7.27 to 7.38), being slightly lower in the shocked cohort (7.31 Vs 7.34, p=0.05). The median lactate levels were 3.1mg/dL (IQR 1.6 to 5.7mg/dL), with significantly more detectable lactate present in those patients requiring inotropes (3.6 Vs 1.8mg/dL, p=0.002).

Median creatinine levels were 117mg/dL (IQR 81 to 183mg/dL) with higher creatinine levels observed in shocked patients (136 Vs 94 mg/dL, p=0.02). Similarly, other renal variables showed a consistent derangement with higher urea levels (8.6 Vs 13.7mg/dL, p=0.0006) and lower urine outputs observed in patients requiring inotropes on day 1 (1000mls Vs 1645mls, p=0.002).

Median bilirubin levels were 15mg/dL (IQR 9 to 31mg/dL) and median AST was 48units/L (IQR 21 to 88units/L). Neither measure of hepatic function was associated with the presence of septic shock.

Shocked patients had a higher INR than those patients not requiring inotropes (1.25 Vs 1.4, p=0.02). However, when other haematological variables were assessed

no difference was detected in the platelet count and APTT between the shocked and non-shocked groups.

On admission to the ICU the median PaO2/FiO2 ratio was 143 mmHg (IQR 94 to 226). However, this measure of the severity of acute respiratory disease was unrelated to the presence or absence of septic shock on admission to the ICU.

Demographics of the shocked and non-shocked patients on admission to the ICU are presented in table 3.1.

Table 3.1: Demographics of the ICU group on day 1

	Shocked	Non-shocked	P value
N	42	20	
Male	26 (61%)	10 (50%)	Ns
Age	72 (57.5 – 59)	64.5 (45 – 74)	Ns
MAP	62.5 (60 – 70)	75 (65 – 80)	0.0009
Lactate	3.6 (2.3 – 6)	1.8 (1.2 – 3)	0.002
SAPS2 score	47 (39 – 59)	35 (28 – 41)	0.0008
SOFA score	9 (8 – 12)	4 (3-5)	<0.0001
MODS	7.5 (5 – 9.25)	5 (3.25 – 6.75)	0.002
Urinary output	1000 (595 – 2000)	1645 (1500 – 2200)	0.002

Results are presented for the two groups; septic shock and severe sepsis on day 1 of ICU stay. Values are either absolute counts with percentages in parenthesis or median values with interquartile range in parenthesis. Age, years. MAP, mmHg. Lactate, mg/dL. Urinary output, mls in 24 hours.

29 (47%) of the ICU patients were admitted under the care of a physician. 33 (53%) of the ICU patients were admitted under the care of a surgeon. The median interval between hospital and ICU admission was 2 days (IQR 0-13 days) with the vast majority (46 patients (74%)) being admitted to the ICU within 24 hours of hospital admission.

Of the surgical ICU patients 14 (42% of the surgical population) underwent a surgical procedure in the 24 hour period prior to ICU admission. In these patients; 11 underwent a laparotomy, one patient underwent an oesphagectomy, one patient underwent a trans urethral resection of prostate and one patient underwent an incision and drainage of an epipidymal abscess.

No relationship was detected between severity of illness and medical or surgical cases. In the surgical patients, a recent surgical procedure (within 24 hours of ICU admission) was unrelated to severity of illness, outcome or any subsequent pattern of cytokine gene expression either on ICU admission or on day 7.

33 (53%) of ICU patients were culture negative in terms of microbiological samples, whereas 8 (13%) of the ICU patients had more than one organism cultured either from different sites or from the same site. An *enterococcus* species was isoloated in 5 cases, *escheria coli* in 5 cases, a *pseudomonas* species in 4 cases, *proteus mirabilis* in 4 cases, a methicillin resistant *staphylococcal aureus* in 3 cases, a sensitive *staphylococcal aureus* in 3 cases, a *streptococcus pneumonia* in 3 cases, a *candida* species in 2 cases, *haemophilus influenza* in 2 cases amd one case each of *neisserria meningitis*, *clostridium perfinges* and a *klebsiella* species. The infecting species was unrelated to severity of illness, outcome or subsequent cytokine gene expression.

Table 3.2: clinical details of the medical/surgical patients in the ICU on day 1

	Medical patients	Surgical patients	p value
N	29	33	
Age	71 (54-78)	69 (63 – 78.5)	0.48
Inotropes	19 (66)	23 (70)	0.79
SOFA score	9 (5.5-11)	8 (5-9.5)	0.45
Death in ICU	5 (17)	12 (36)	0.15
Gram negative organism isolated	5 (42)	7 (44)	1.0

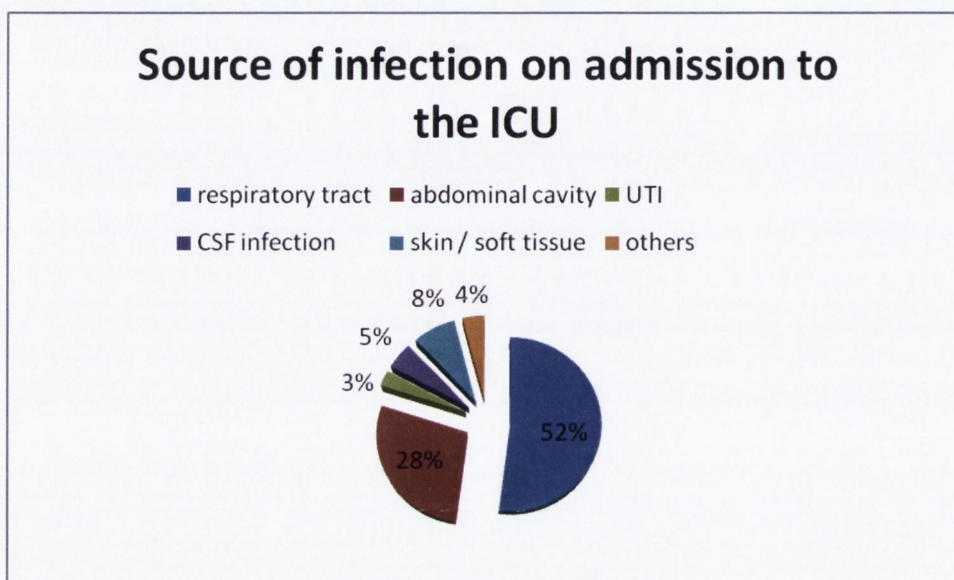
Results are presented for the two groups; medical and surgical patients on day 1 of ICU stay. Values are either absolute counts with percentages in parenthesis or median values with interquartile range in parenthesis. Age, years.

On admission to the ICU the most common infective source was the respiratory tract accounting for 52% of all infections. Abdominal infections were the second most prevelant at 28%, followed by skin and soft tissue infections in 8%, central nervous system infections in 5% and urinary tract infections in 3% (figure 3.1). 33 (53%) patients were culture negative for their admitting infective insult. Where an organism was isolated, gram-positive organisms were the most common (66%), followed by gram-negative organisms (43%) and fungal pathogens (7%).

The mean SAPS 2 score was 44 (SD 16), the mean SOFA score 8 (SD 3.8) and the mean MODS was 6.7 (SD 3.2). Interestingly, lower SAPS2 scores were associated with gram-negative infections when compared to gram-positive and fungal infections in those patients having positive cultures (37 Vs 53, p= 0.014). This association remained when analysing the association with the MODS (5.7 Vs 8.9,

p=0.005) and whilst a similar trend was present when the SOFA score was used the analysis failed to reach statistical significance (7.3 Vs 10, p= 0.06).

Figure 3.1



Frequency of the source of infectious insults in ICU patients. This pie chart depicts the breakdown of patients in the ICU by the source of their infectious insult necessitating their ICU admission.

3.1.2 Description of clinical and biochemical variables on day 7 of ICU stay

49 (79%) patients had blood samples drawn and analysed on day 7 of ICU stay. 39 (63%) patients had blood samples drawn and analysed at both time points.

On day 7, 15 (28%) patients were characterised as having septic shock with the remaining 38 (72%) fulfilling the criteria for severe sepsis only. In a similar fashion to day 1, the majority of patients requiring inotropes received noradrenaline (14) with a minority of patients (1) receiving adrenaline alone and no patients receiving both. Where noradrenaline was being administered on day 7 the median noradrenaline dosage was 11mcg/min (IQR 3.8 to 18.5 mcg/min).

On day 7 in ICU the median MAP was 80mmHg (IQR 70 to 90 mmHg). Again, the MAP was significantly lower in those patients requiring inotropes (70 mmHg Vs 81.5 mmHg, p=0.002). Mean CVP was 11mmHg (SD 3.7) and there was no detectable difference in shocked and non-shocked patients with respect to their CVP (11 mmHg Vs 11mmHg, p=0.98).

The following measures were made on day 7 of ICU stay unless otherwise stated.

The median pH was 7.42 (IQR 7.36-7.45) with patients requiring inotropes having a significantly lower pH than those not requiring inotropes (7.37 Vs 7.44, p=0.0002). Median lactate levels were 1.6mg/dL (IQR 1.1 to 2.2mg/dL) with the shocked group having significantly more detectable lactate than those not requiring inotropes (1.4 Vs 2.1mg/dL, p=0.017).

Median creatinine levels were 94mg/dL (IQR 70 to 145mg/dL) and higher creatinine levels were observed in shocked patients (131 Vs 78mg/dL, p=0.04). Median urinary output over these 24 hours was 1600mls (IQR 588mls to 2373mls) with higher urinary output observed in the group of patients not requiring inotropes (1920 Vs 550, p=0.0002). However, the remaining renal variable recorded, urea failed to distinguish between the shocked and non-shocked groups at this time point (p=0.26).

Median bilirubin levels were 15mg/dL (IQR 9 to 30.5mg/dL) and median AST levels were 44units/L (IQR 26 to 65units/L). Neither measurement was associated with the use of inotropes (p=0.09 and p=0.8 respectively).

The median INR was 1.1 (IQR 1.0 to 1.3) and was higher in shocked patients (1.2 Vs 1.1, p=0.03). However, the remaining haematological variables collected,

platelet count and APTT, did not differentiate between shocked and non-shocked groups.

The median PaO2/FiO2 ratio was 191 mmHg (IQR 153 to 280). Patients with lower ratios were more likely to be inotrope dependant (212 Vs 180, p=0.02).

The median MODS was 6 (IQR 2 to 8) and the median SOFA score was 5 (IQR 3 to 10). However, the association of organ failure score and the classification of the infectious organism did not remain statistically significant at this time point.

Demographics for the ICU group on day 7 are presented in table 3.3.

Table 3.3: Demographics of the ICU group on day 7

	Shocked	**Non-shocked**	**p value**
N	15	38	
Male	11 (73%)	10 (47%)	Ns
Age	69 (54 – 75)	72 (57 – 78)	Ns
MAP	70 (70-70)	83 (80 – 90)	0.002
Lactate	2.1 (1.6 – 3.2)	1.3 (1.0 – 1.95)	0.01
SOFA score	11 (10 – 15)	4 (0 – 6)	<0.0001
MODS	9 (7 – 12)	3 (1 – 6)	<0.0001
Urinary output	550 (100 – 1395)	1960 (1488 – 2815)	0.0002

Results are presented for the two groups; septic shock and severe sepsis on day 7 of ICU stay. Values are either absolute counts with percentages in parenthesis or median values with interquartile range in parenthesis. Age, years. MAP, mmHg. Lactate, mg/dL. Urinary output, mls in 24 hours. ns, non significant.

3.1.3 Trend in clinical variables over time in the ICU group

As we have more than 30 samples at each time point we may assume that the sampling distribution for the group means conforms to a Normal distribution. Therefore, the following comparisons utilise the paired t-test to assess changes in the variable from admission to ICU to day 7 of ICU stay.

Over the course of the first week in ICU the MAP rose an average of 13mmHg ($p< 0.0001$) while the CVP remained unchanged ($p=0.92$).

Detectable lactate fell an average of 1.8mg/dL ($p<0.0001$) whilst pH rose by a mean of 0.08 ($p=0.0003$).

Renal parameters showed similar improvements with urea levels falling by an average of 3.6mg/dL ($p=0.046$). There was also a small non-significant fall in creatinine levels over the initial week (mean difference –29mg/dL, $p=0.08$). However, there was no change in the 24-hour urinary output during this period ($p=0.24$).

Hepatic parameters remained static with bilirubin levels ($p=0.52$) and AST ($p=0.92$) unchanged over the course of the week.

Whilst the INR improved by a mean of 0.49 ($p=0.001$), other coagulation parameter such as platelets and APTT were unchanged ($p=0.34$ and $p=0.054$ respectively).

The PaO2/FiO2 ratio improved by an average of 57mmHg in ICU patients over the course of the initial week ($p=0.0009$).

These changes are reflected by an average improvement in the SOFA score of 1.6 ($p=0.008$) and in the MODS of 1.1 ($p=0.037$) from admission to day 7.

3.1.4 Association of clinical variables to outcome

Overall mortality was 27.4%. Clinical and demographic variables that were thought could plausibly be associated with outcome following a septic insult in an ICU setting were tested for their association with the outcome variable. This was prospectively defined as either death during the ICU stay or discharge from the ICU.

Patient age on admission to ICU had no influence on outcome (p=0.59). Similarly, MAP and CVP on admission (p=0.33 and p=0.21 respectively) and again on day 7 (p=0.67 and p=0.98) were not associated with outcome. There was also no detectable association between the season of admission to the ICU and outcome (p=0.94).

The use of inotropes on day 1 was associated with outcome, with 16 of 17 patients who subsequently died requiring inotropes on admission to ICU compared with 27 of 46 patients who were subsequently discharged from ICU (p=0.007). In those patients requiring inotropes the maximum dose of noradrenaline received on day 1 was not associated with a poor outcome. In patients still alive on day 7, but who subsequently died, 5 of these 8 patients required inotropes on day 7 while 10 of 46 patients who subsequently survived required inotropes (p=0.03).

Of the renal parameters only a greater 24-hour urinary output on day 1 was associated with an increased survival (p=0.02). On day 7 a similar trend persisted which did not reach significance (p=0.07).

None of the metabolic parameters were associated with outcome on day 1. However, on day 7 those patients who remained acidotic were more likely to have a poor outcome (p=0.004).

No association was detected between type of infectious organism and outcome. However, those patients who mounted a lesser white cell response on day 1

were more likely to have a poor outcome (p=0.02). This association did not persist on day 7.

Using the standard organ failure assessment tools on day 1, the SAPS2 and the SOFA scores were higher in patients who subsequently died (p=0.02 and p=0.004, respectively). The MODS, however, failed to reach statistical significance on day 1 although a similar trend was evident (p=0.06). On day 7 both the SOFA score and the MODS predicted mortality (p=0.01 and p=0.008).

Table 3.4: Factors associated with outcome in the ICU group

	Death (n=17)	Survival (n=45)	p value
Age	69 (57 – 79.5)	71 (54 – 78)	ns
MAP day 1	65 (60 –70)	65 (60 – 79)	ns
MAP day 7	80 (70 – 90)	80 (70 –90)	ns
Inotropes day 1	16 (94)	27 (58)	0.007
Inotropes day 7	5 (62.5)	10 (22.2)	0.03
Urine output day 1	935 (300 – 1848)	1500 (950 – 2000)	0.02
Urine output day 7	677 (225 – 1399)	1800 (850 – 2600)	0.07
pH day 1	7.32 (7.27 – 7.39)	7.31 (7.27 – 7.38)	ns
pH day 7	7.32 (7.24 – 7.40)	7.43 (7.38 – 7.45)	0.004
WCC day 1	10.7 (3.4 – 15.75)	18 (11.1 – 23)	0.02
WCC day 7	13.9 (8.5 – 18.6)	13.1 (9.5 – 22.3)	ns
SAPS2	49 (40.5 – 62.5)	40 (33 – 50.5)	0.02
SOFA day 1	9 (9 –12.5)	7 (4 – 9.5)	0.004
SOFA day 7	10.5 (8.5 – 11.8)	4.5 (1.8 – 9)	0.01
MODS day 1	7 (5.5 –10)	6 (4 – 8)	0.06
MODS day 7	8 (7 – 10.75)	4 (1 – 7)	0.008

Results are presented for the two outcome groups. Values are either absolute counts with percentages in parenthesis or median values with interquartile range in parenthesis. Age, years. MAP, mmHg. Urinary output, mls in 24 hours. ns, non significant. WCC, white cell count. SAPS, simplified acute physiology score. SOFA, sequential organ failure assessment. MODS, multiple organ dysfunction score.

3.2 Bacteraemic group

3.2.1 General description

The bacteraemia group comprised 10 patients, 4 male and 6 female. The median age was 82 (IQR 68.5 to 86 years). All patients, by virtue of the inclusion criteria, had a gram-negative bacillus (GNB) isolated on blood cultures. The source of the bacteraemia was deemed to be the respiratory tract in 4 (40%), the urinary tract in 4 (40%) and the abdominal cavity in 2 (20%).

Six (60%) patients were classified as medical admissions and 4 (40%) as surgical admissions.

The median WCC in this group was 9.6 (IQR 7 to 13) at the time of blood sampling. The median SOFA score was 0.5 (IQR 0 to 3) and the median MODS was 1 (IQR, 0 to 2.3) and was assessed at the time of blood sampling using the same criteria as in the ICU group.

3.2.2 Comparison of bacteraemia and ICU groups

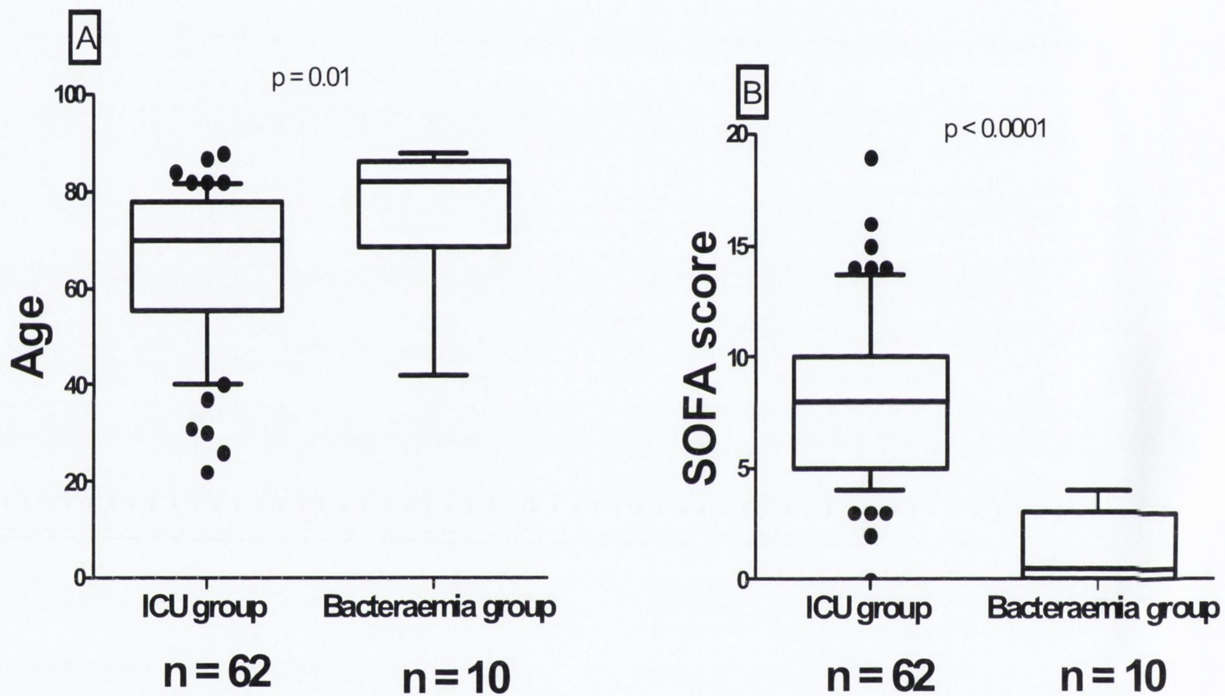
Patients in the bacteraemic group were significantly older than those in the ICU group ($p=0.01$, figure 3.2A).

SOFA scores on admission to ICU were found to be higher than SOFA scores in the bacteraemia group at the time of blood sampling ($p< 0.0001$, figure 3.2B). Similarly, MODS on ICU admission were higher than MODS in the bacteraemia group at the time of blood sampling (6 Vs 1, $p< 0.0001$, table 3.5).

The WCC was non-significantly lower in the bacteraemia group at the time of blood sampling when compared to the ICU group on admission (9.6 Vs 15.6, $p=0.08$). The only difference detectable between groups as regards site of the original infection

was for UTIs. In four out of ten bacteraemic patients the urinary tract was deemed to be the source of the infection in comparison to 2 out of 60 ICU patients (p=0.003, table 3.5).

Figure 3.2



Comparison of the ICU and the non-critically ill bacteraemic group. Values, represented as box plots, are median, quartiles 25% to 75% (box), and centiles 10% to 90% (whiskers).

Table 3.5: ICU group Vs Bacteraemia group

	ICU group	Bacteraemic group	p value
N	62	10	
Male	34 (55%)	4 (40%)	
Age	70 (55.5 – 78)	82 (68.5 – 86)	0.01
SOFA score	8 (5 – 10)	0.5 (0 – 3)	<0.0001
MODS	6 (4 – 9)	1 (0 – 2.25)	<0.0001
Site of infection			
Urinary tract	2 (3%)	4 (40%)	0.003

Results are presented for the two groups; ICU group and bacteraemic group. Values are either absolute counts with percentages in parenthesis or median values with interquartile range in parenthesis. Values for the ICU group are for day 1 of ICU stay. Age, years. Ns, non significant

3.3 Discussion

As sepsis is classically such a heterogenous disorder, it is necessary to characterise our patient population in order to allow meaningful comparisons with other studies and also to properly evaluate the comparisons we are making between septic and bacteraemic groups.

The largest recent epidemiological study of an ICU population comes from a study conducted in 2001 (Angus et al., 2001) which examined 192,980 patients admitted to American ICUs with a diagnosis of severe sepsis. In this case the International Classification of Diseases, ninth revision, clinical modification (ICD-9-CM) criteria were used to characterise patients with infections and specific criteria were also used to identify organ dysfunction. This study described a mean patient age of 63.8 years in a cohort of patients that was 49.6% male. They identified the source of the infection as being the respiratory system in the majority (44%) of cases, with the genitourinary system being identified as the source in 9.1% of cases and the abdominal cavity in 8.6% of cases. The overall mortality was 28.6%.

This paper is, at present, one of the most widely recognised and referenced epidemiological papers in the critical care community. Overall, our demographic and clinical data is very similar to the data described by Angus et al. However, our patient cohort has a greater propensity for the source of the infection to originate from the respiratory tract (52% Vs 44%) and abdominal cavity (28% Vs 8.6%) and the genitourinary tract was less likely to be the source of the infectious insult (3% Vs 9.1%).

This discordant epidemiological data may be explained through an analysis of the inclusion criteria. Whereas we studied only an adult population, Angus et al included paediatric patients. Additionally, HIV patients, those with haematological

malignancies and other immunocomprimised groups, which were excluded from our study, were included in this epidemiological study. The addition of these groups of patients is likely to influence to some degree the source of infectious insult.

Additionally, an important omission from the study by Angus et al was the proportion of patients admitted with or subsequently developing septic shock. The diagnosis of septic shock as opposed to severe sepsis is particularly ominous and is regarded as the most extreme end of the spectrum of disease that is sepsis.

However, a recent US interventional placebo-control study examining the safety profile of an anti-TNFα compound reported demographic and clinical details including the incidence of septic shock in their cohort (Rice et al., 2006). The study drug was administered within 36 hours of ICU admission and the data collected are described as baseline demographics. Additionally, this study specifically excluded HIV patients, patients with haematological malignancies and those receiving chemotherapy.

The authors described a mean age in the treatment group (43 patients) of 52 years and 65% of patients were male whilst in the control group (38 patients) the mean age was 59 years with 61% of the patients being male. In the treatment arm, 79% of the patients were classified as shocked prior to receiving the study medication and in the control group 87% of the patients had septic shock over the same observational period. In 51% of the treatment group the respiratory tract was deemed the source of the infectious insult compared with 37% of the control group. Whilst no further exact data are available on additional sources of infection an analysis of the graphical data presented suggests that the abdominal cavity was the next most common source of infectious organisms.

Descriptive data were collected at the time of administration of the study medication, which was within 36 hours of the development of severe sepsis. Therefore, it is reasonable to compare these data with the day 1 data collected on our patient group. Our patient group on admission to the ICU appear reasonably similar to both arms of this interventional trial. We note that 68% of our patients were shocked on admission to the ICU with, as stated above, the respiratory tract being the source of the pathogen in 52% of all cases. The relatively small number of patients in both studies may contribute to any of the minor differences noted. Additionally, the inclusion criteria differ with either shock or dysfunction of two or more organs being necessary for inclusion to the interventional study presented above whilst dysfunction of a single organ was deemed sufficient for inclusion to our study.

Based on these data we feel that the cohort of patients we describe is typical of an ICU population in the developed world. We failed to find significant differences between our population and other previously described ICU populations with severe sepsis and septic shock. Consequently, the results that follow are applicable to ICU populations in other similar centers.

Some of the results of the analysis of the demographic and clinical data warrant closer analysis. The recruitment period of the study deliberately stretched over 12 months to examine for a seasonal influence on the severity of the infectious insult. Theoretically, if the recruitment period was shortened there is the potential for an unrecognised enhanced severity of infection in a particular season to influence the results. Furthermore, it was felt that certain sources of infections might be more prevalent at a particular time of year. The relevance of this lies in the perceived potential of studies conducted over a winter period to recruit an excess of elderly patients with respiratory infections. Analysis of the results reveals that the time of

year a patient is admitted to the ICU does not influence that infectious source, severity of the infection or mortality. Additionally, we did not observe any relationship between patient age and the season of ICU admission. Therefore, we feel that a shorter recruitment period would not result in the collection of a non-random sample by virtue of seasonal variation in ICU admissions in patients with severe sepsis.

We noted with interest that in the septic shock group, despite treatment with inotropes to attempt to achieve a target MAP of 60mm Hg, blood pressure remains significantly lower in this group than in the non-shocked group. Treatment with inotropes, however, was deemed successful in raising the MAP to this target level in the majority of shocked patients as the median MAP was 63mmHg and 70mmHg on day 1 and day 7 respectively in the group with septic shock. Whilst lower than pressures achieved in the non-shocked group, these levels are usually deemed adequate for organ perfusion by the majority of ICU clinicians.

Additionally, we can comment on the adequacy of fluid resuscitation in the group with septic shock by an assessment of the central venous filling pressures. The CVP on both day 1 and day 7 was significantly higher in the septic shock group, which suggests that this group were more aggressively fluid resuscitated. Therefore, from this limited information, it appears that the septic shock group were adequately treated and did not have a poorer outcome as a result of sub optimal resuscitation.

However, despite this appropriate treatment the shocked group displayed signs of tissue hypoperfusion. This can be observed in the differences in the recorded metabolic variables between the groups. Lactate was higher and pH lower in the shocked group at both time points. This implies that despite appropriate treatment with inotropes and fluids, a group of patients proceeded to develop more severe disease as is manifest by a persistent lactic acidosis presumably secondary to tissue

dysoxia. The differences noted in other markers of organ function described earlier between the shocked and non-shocked groups appear to confirm this finding.

Interestingly, on admission to the ICU, severity of disease was less in patients with a gram-negative infection. This has not previously been reported and suggests that in our cohort gram-negative infections were possibly less virulent than other infecting organisms.

Also, it is notable that the median values of the clinical variables tended to improve over the course of the initial week in ICU. This may reflect both the appropriateness of clinical care received and a tendency for these patients to improve over time when adequately supported. However, these data may be biased as they do not take into account the 12 patients who died prior to contributing to the day 7 data. Furthermore, we noted that both the median MODS and SOFA score improved over this initial week, but again these data must be viewed with the same caveats.

The MODS scoring system was the first organ failure system to be introduced that quantified the degree of organ dysfunction as opposed to simply counting the number of failing organs. Both the SOFA and the SAPS2 scoring systems have been heralded as providing improvements in the prognostic powers of the original tests. These scoring systems are currently in widespread use due to their simplicity, reliability and reproducibility. It has previously been shown that there is a clear correlation between the total organ failure score and mortality rate in ICU patients (Vincent, 2006).

We can confirm these previous findings in our population by reporting that on admission to the ICU, both SAPS 2 and SOFA scores could predict subsequent mortality whilst the MODS failed to distinguish survivors from non-survivors. This may be indicative of the improvements made in the developments of the scoring

systems, particularly to the cardiovascular variable. The MODS uses a formula comprising the heart rate, blood pressure and CVP whereas the SOFA score is felt to refine this by assessing the dose of inotropic medication required. Although the SAPS2 score uses only the systolic blood pressure and heart rate to compute a cardiovascular variable, it also assesses additional variables such as age, chronic diseases, electrolyte concentrations and metabolic variables.

Interestingly, we demonstrate that the prognostic power of the MODS and SOFA score both increase when used later in the time course of the disease. On day 7 both scores more accurately predict subsequent non-survivors than they do on admission to the ICU.

As regards individual clinical and demographic variables we noted that inotrope requirements, reduced urinary output, acidosis and a reduced WCC were associated with mortality. It is somewhat surprising that patient age was unrelated to outcome as this has been clearly described in the past (Angus et al., 2001). The relatively small numbers recruited to this study may account for this.

Inotrope requirements, poor urinary output and acidosis are all factors that have been previously recognised as being strong indicators of a poor outcome in the ICU setting (Martin et al., 2000). Whilst a transient leukopenia has been described as being as associated with the onset of septic shock (Damas et al., 1997) and is recognised as a poor prognostic sign, this moderate reduction in the WCC has not been previously reported. It has been hypothesised that the previously reported leukopenia may represent a mechanism of subversion of immunity by pathogens. In our cohort, the failure to mount an adequate white cell response to an infecting organism may represent the lack of an appropriate inflammatory response in these patients that eventually succumb as a result of their infection.

3.4 Conclusion

In conclusion, we have recruited a cohort of patients with severe sepsis that closely resembles other previously described patient groups presenting with the same diagnosis with respect to severity of disease, source of infection, age and mortality. The clinical factors and demographic variables described in this study that are associated with the eventual outcome of these patients are factors that have been well recognised in the past to be associated with a poor prognosis. We feel that as a result the subsequent results are applicable to other critical care centers in the developed world containing a similar heterogenous mix of patients.

Chapter 4 - Results (2): Description of the distal effector cytokines in septic patients and comparisons between patient groups

4.1 Introduction

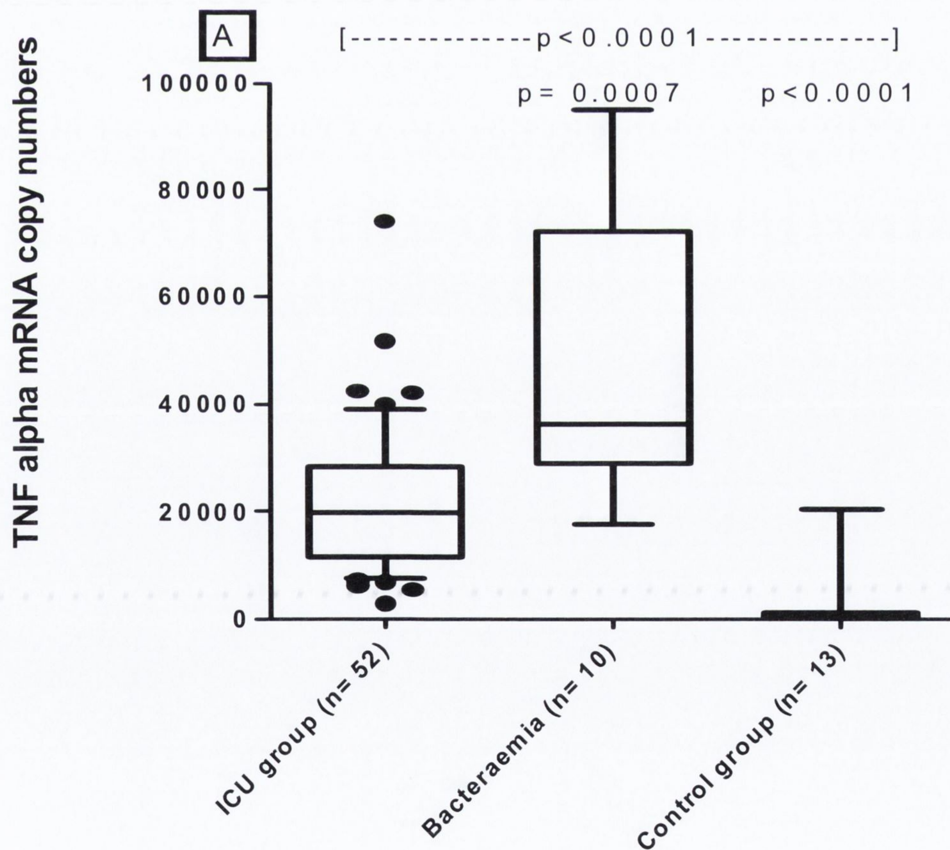
For the purposes of this discussion the cytokines assayed by QRT-PCR will be grouped and discussed separately. Cytokines will be referred to as proximal / regulatory and distal / effector cytokines. In practice there is a significant overlap between cytokines that have a primary effect on end organ and cellular function versus those whose major effects are promotion or inhibition of the production of effector cytokines. This is easily observed in the case of IFNγ, whose production further polarises cells towards a Th1 response as well as having effects on bactericidal function. Therefore, whilst this segregation is somewhat arbitrary it facilitates the logical analysis of the data set.

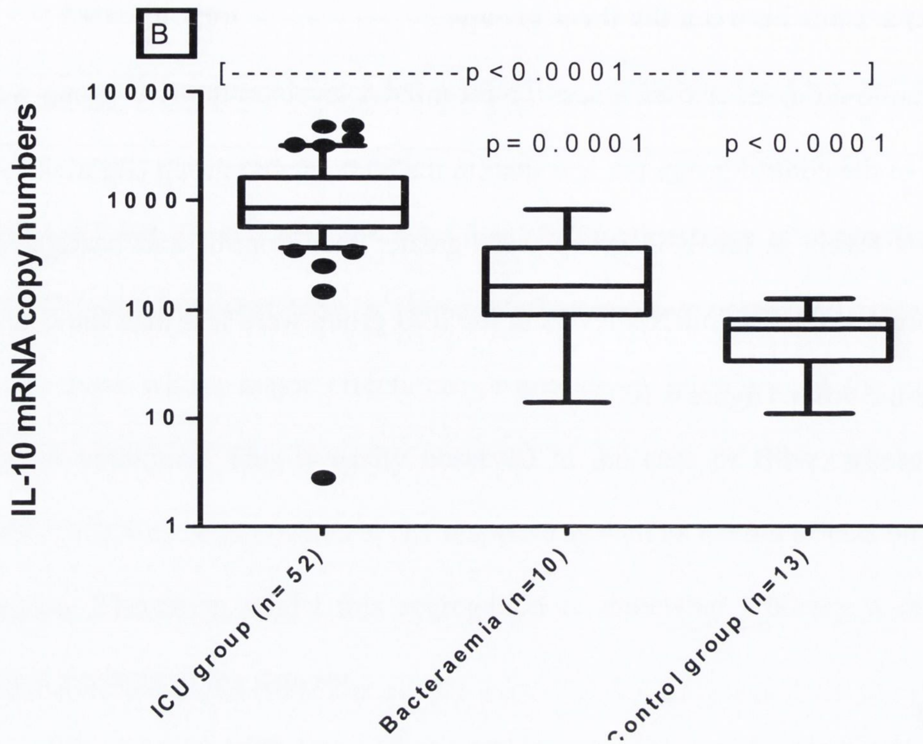
This section will deal with the effector cytokines and includes data on TNFα, IL-10 and IFNγ. Throughout the results section cytokine copy numbers are expressed as mRNA copy numbers standardised per 10 million β actin mRNA copies.

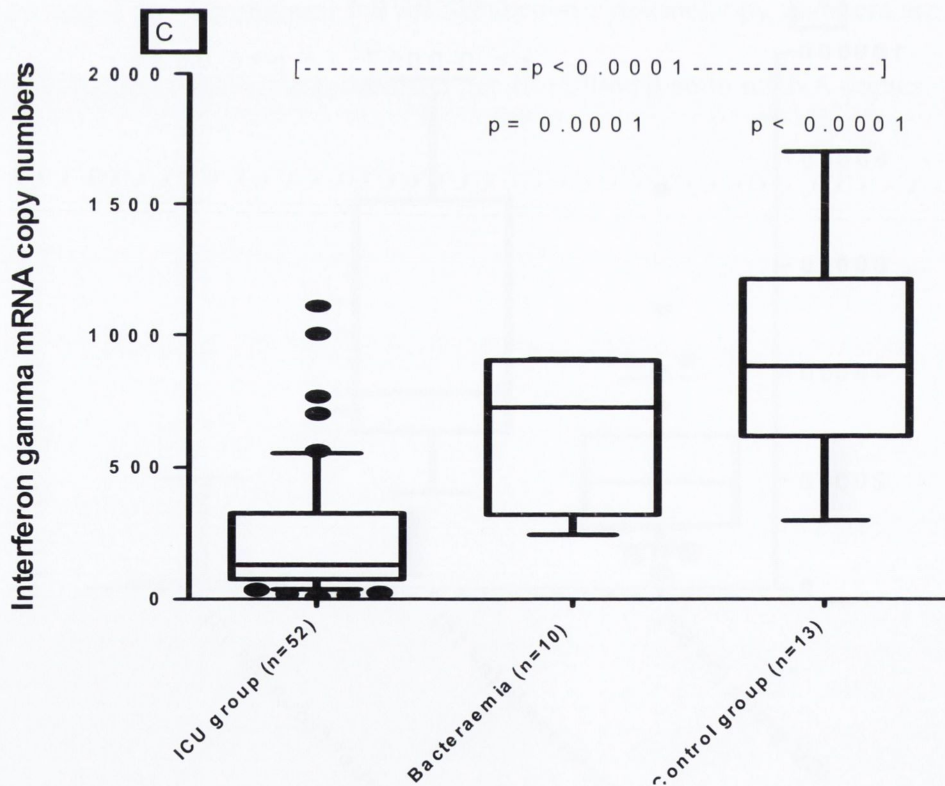
4.2 Comparisons between the three groups

On the first day of critical illness TNFα mRNA levels in the ICU group were greater than in the control group but less than in the bacteraemic group (figure 4.1A). IL-10 mRNA levels in the ICU group were greater than control and bacteraemic groups (figure 4.1B). IFNγ mRNA levels in the ICU group were less than the control and bacteraemic group (figure 4.1C).

Figure 4.1







Proximal cytokine mRNA levels in the ICU group on day 1 of ICU admission, in the non-critically ill bacteraemaic group and the control group. Values, represented as box plots, are median, quartiles 25% to 75% (box), and centiles 10% to 90% (whiskers). Individual p values are for comparison with the ICU group. The p value at the top of the figure represents a 3-group comparison.

4.3 Multivariate group comparison

A multivariate logistic regression model of patients in the bacteraemic and ICU groups was constructed which included data for TNFα, IFNγ and IL-10. This model had an R^2 value of 0.63, with a receiver operator characteristic curve value of 0.96. From this model the respective probabilities of belonging to the bacteraemic and ICU groups was determined for each patient and the ratio of these probabilities was considered as a relative risk for outcome in response to infection. This relative risk scoring system could be used to identify the bacteraemic group with 100% specificity and 70% sensitivity, or identify the ICU group with 100% specificity and 94% sensitivity.

4.4 ICU group

4.4.1 Description of the effector cytokines on Day 1

On day 1, in the ICU cohort, median TNFα mRNA levels were 19867 (IQR 11546 – 28271). Median IL-10 mRNA levels were 852 (IQR 573 – 1639) and median IFNγ mRNA levels were 132 (IQR 77 –351). Levels of these cytokines did not distinguish between those patients with severe sepsis and those with septic shock on day 1. Cytokine mRNA levels on day 1 were not associated with any of the organ failure scores, extent of lung injury as assessed by the PaO2/FiO2 ratio, lactate levels or with the type of infecting organism (gram positive, gram negative or fungal).

4.4.2 Description of the effector cytokines on Day 7

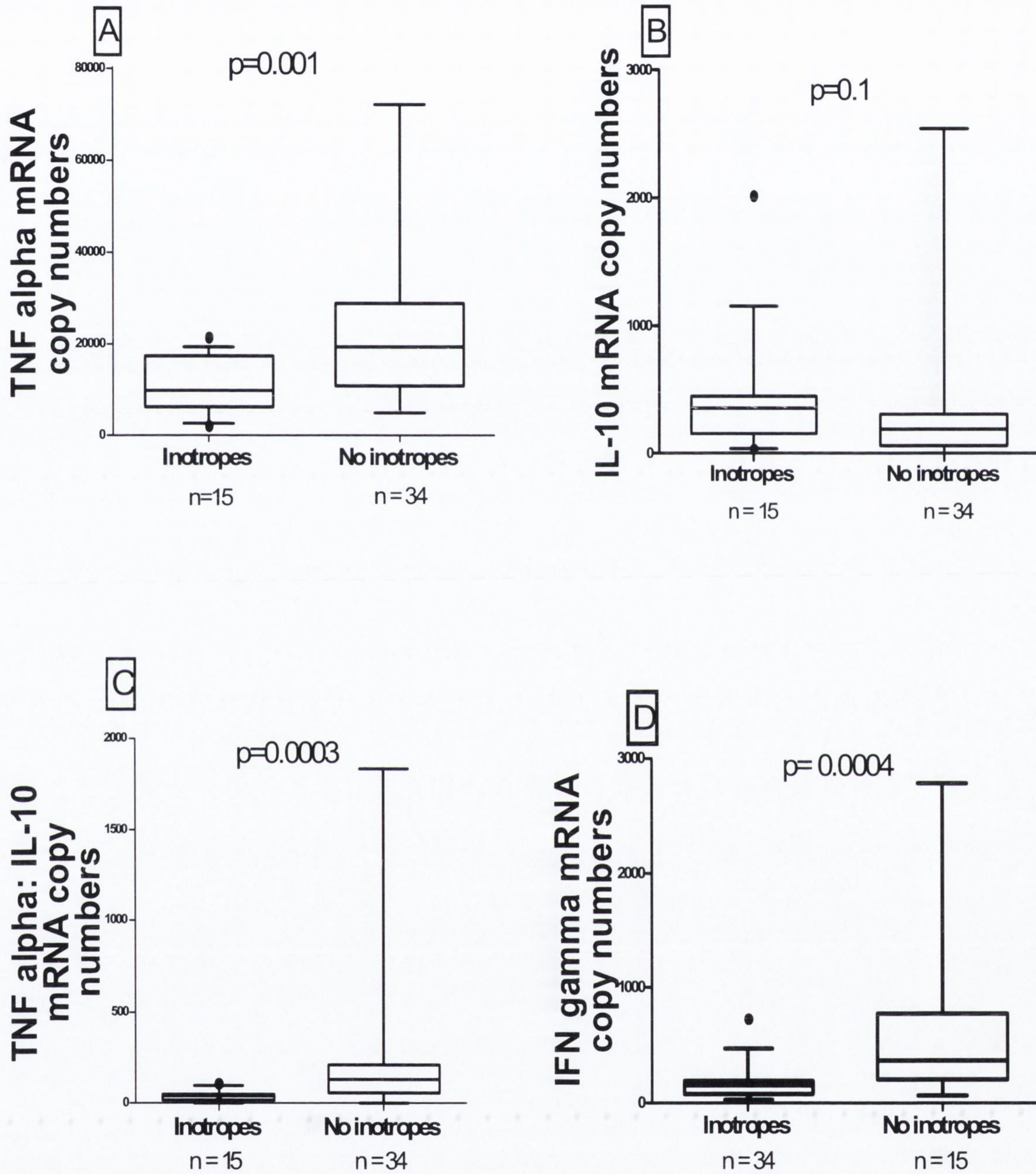
On day 7, median TNFα mRNA levels were 15242 (IQR 9342 –23819). Levels were higher in those patients not requiring inotropes when compared with patients with septic shock ($p=0.001$, figure 4.2A). TNFα mRNA levels were inversely related to both SOFA scores ($p<0.0001$, figure 4.3) and MODS scores ($n=49$, spearman's rho 0.45, $p=0.0015$). There was also a borderline inverse correlation between lactate levels and TNFα mRNA levels ($n=42$, spearman's rho –0.31, $p=0.04$). There was no association between TNFα mRNA levels on day 7 and extent of lung injury or with the type of infecting organism.

On day 7, median IL-10 levels were 219 (IQR 96 – 373). Although there was a trend for patients with higher IL-10 mRNA levels to require inotropes, this difference was not statistically significant (figure 4.2B). IL-10 mRNA levels on day 7 were not associated with any of the organ failure scores, extent of lung injury, lactate levels or with the type of infecting organism.

On day 7, the median TNFα / IL10 ratio was 88 (IQR 37 – 182). Patients requiring inotropes on day 7 had lesser TNFα / IL10 ratios than those patients not requiring inotropes ($p=0.0003$, figure 4.2C). However, this ratio was not associated with any of the organ failure scores, extent of lung injury, lactate levels or with the type of infecting organism.

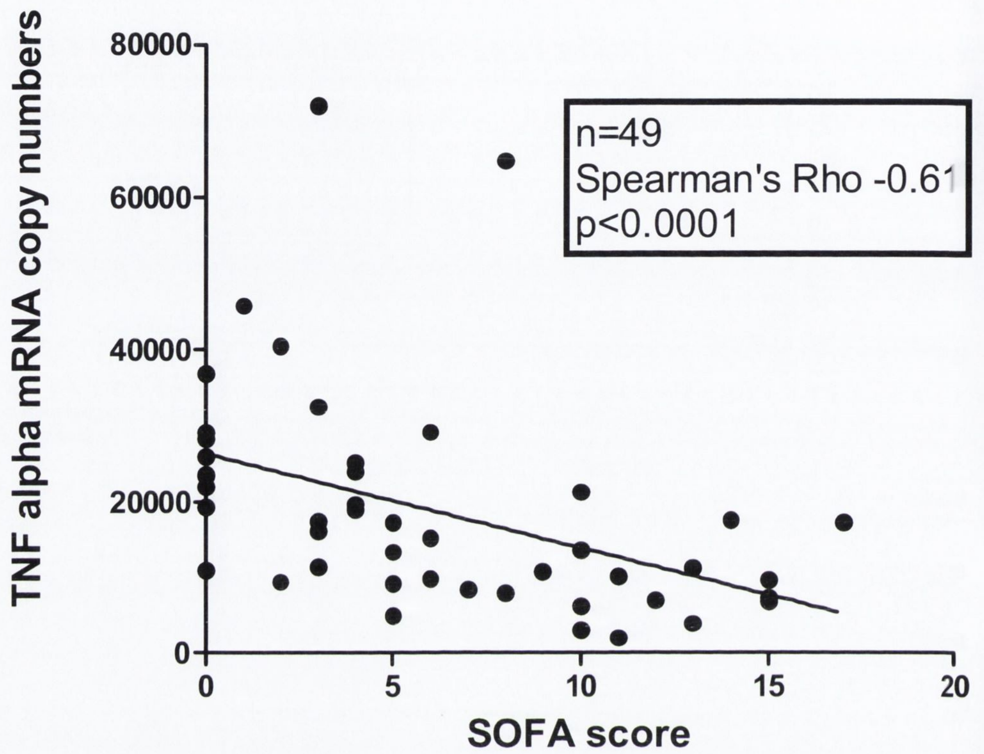
On day 7 median IFNγ levels were 257 (IQR 139 – 637). Levels were lower in those patients requiring inotropes on day 7 in comparison to patients not requiring inotropes ($p=0.0004$, figure 4.2D). Levels were inversely related to the SOFA score ($p=0.02$) and the MODS score ($p=0.02$). There was no association detected between IFNγ levels and the extent of lung injury, lactate levels or the type of infecting organism.

Figure 4.2



Levels of proximal cytokines in patients who required inotropes and in those independent of inotropes on day 7 of ICU stay. Values, represented as box plots, are median, quartiles 25% to 75% (box), and centiles 10% to 90% (whiskers).

Figure 4.3



Correlation between TNFα mRNA on day 7 of ICU stay and SOFA score on day 7 in the ICU group.

4.4.3 Change in mRNA levels over time

The 39 ICU patients with RNA analysis available at both time points were assessed for a change in mRNA levels over time. In these patients there was no change in TNFα mRNA levels over the seven days of critical illness, whereas IL-10 mRNA levels decreased and IFNγ mRNA levels increased (table 4.1).

Table 4.1: Changes in Cytokine mRNA levels over 7 days in ICU patients.

Cytokine	**N**	**Day 7 minus day 1 mRNA levels**	**P value**
TNFα	39	0.02 (-0.24 to 0.18)	0.35
IL-10	39	-0.51 (-1.48 to –0.30)	0.001
IFNγ	39	0.39 (-0.04 to 0.76)	0.001

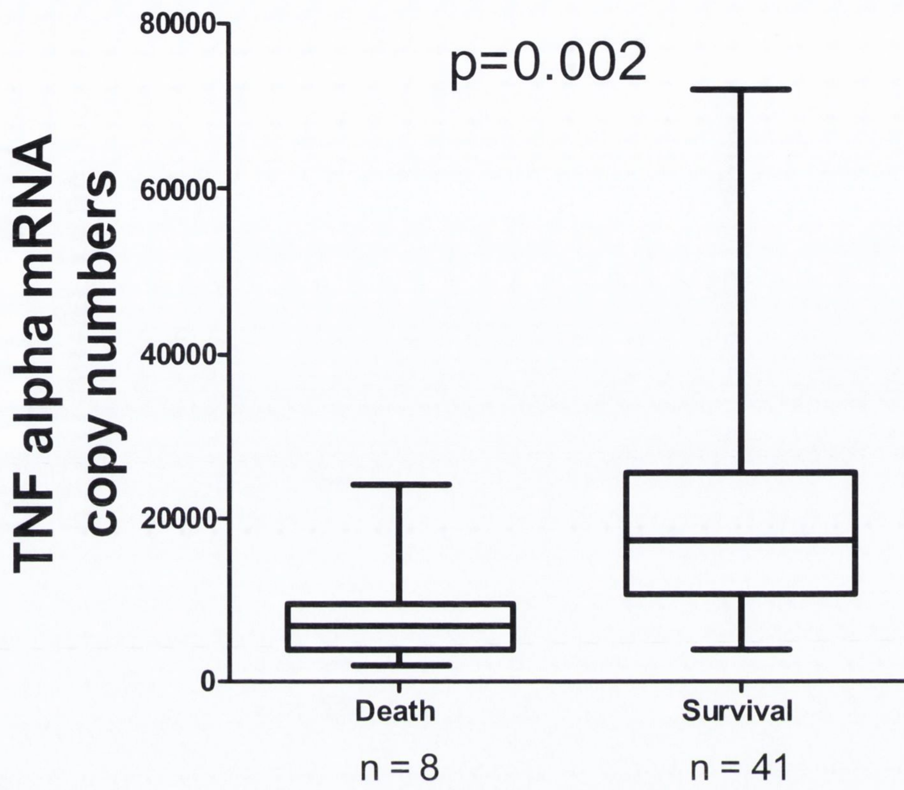
Results are expressed as differences in log mRNA copy numbers per 10^7 β actin mRNA copies when day 7 values are subtracted from day 1 values.
All values are expressed as median difference with interquartile range. Analysis is by Wilcoxon rank sign test.

4.4.4 Relationship to mortality

Seventeen of the 62 ICU patients (27%) died during the course of their ICU stay. Of the 52 patients that were analysed for mRNA levels on the first day of critical illness, 13 died prior to ICU discharge. There was a non-significant trend towards greater IL-10 mRNA levels (1262, (721 – 3169) versus 776 (539 – 1353); $p = 0.1$) and lesser IFNγ mRNA levels (112 (70 – 140) versus 180 (76 – 411); $p = 0.07$) in non-survivors when compared with survivors. However, when ICU patients were dichotomised between those in the highest quartile of IFNγ mRNA production on day 1 and the remainder, no deaths occurred in the highest quartile group, whereas 13 of the other 39 patients died ($p = 0.02$).

Eight of the 49 ICU patients who had blood samples analysed for mRNA production after 7 days of critical illness died prior to ICU discharge. These 8 non-survivors had lesser TNFα mRNA levels (Figure 4.4).

Figure 4.4



Levels of TNFα mRNA in survivors and non-survivors of sepsis measured on day 7 of ICU stay. Values, represented as box plots, are median, quartiles 25% to 75% (box), and centiles 10% to 90% (whiskers).

A cluster analysis (JMP software), which included ICU data for day 7 TNFα and IFNγ mRNA levels, was performed and a cluster of ICU patients with reduced TNFα and IFNγ mRNA levels was characterised. Patients in this cluster were more likely to be shocked on day 7 of critical illness, had greater SOFA scores and had a significantly higher mortality (Table 4.2).

Table 4.2: Cluster analysis of TNFα and IFNγ mRNA levels in the ICU group on Day 7 of critical illness

Cluster	**1**	**2**	**p value**
N	31	18	
TNFα	21379 (16218-28840)	6760 (2884-10715)	0.0001
INFγ	407 (190-794)	158 (93 – 229)	0.0004
Shock	4 (13%)	11 (61%)	0.0009
SOFA	3 (0 – 6)	10 (8 – 13)	0.0001
Outcome (death)	2 (6.5%)	6 (33%)	0.04

Results are expressed as mRNA copy numbers per 10^7 β actin mRNA copies. All continuous variables are quoted as median with interquartile ranges. All comparisons are by Wilcoxin Rank Sum test.

4.5 Discussion

We have observed that patient response to infection was associated with distinct patterns of cytokine mRNA production. More IFNγ and TNFα mRNA was detected in the bacteraemia group when compared to the ICU group. Lesser IFNγ and TNFα gene expression was associated with both the occurrence of severe sepsis in response to infection and also with poor outcome in these ICU patients.

IL-10, a potent anti inflammatory cytokine and a direct inhibitor of macrophage function, exerts anti inflammatory properties via inhibition of NF-κB nuclear translocation and thereby inhibiting pro-inflammatory cytokine gene expression (Moore et al., 2001). IL-10 mRNA levels were greatest in patients with severe sepsis and less in patients with bacteraemia who did not develop critical illness. This pattern of IL-10 gene expression may account for the relatively deficient IFNγ production in patients with sepsis, as IFNγ gene expression in antigen presenting cells may be inhibited by IL-10 (Fiorentino et al., 1991). Furthermore, exogenous IL-10 is reported to inhibit IFNγ production in isolated monocytes from patients with sepsis (Bjerre et al., 2004).

The Th1 cellular response is generally induced by, and particularly effective against, intracellular pathogens and those that activate macrophages and natural killer (NK) cells. This response is associated with enhanced cell-mediated immunity and is therefore the appropriate response expected in the face of most common septic insults. Lymphocytes demonstrating a Th1 response pattern characteristically produce IFNγ, thereby optimising the bactericidal activity of phagocytes (Boehm et al., 1997).

In contrast Th2 biased responses are classically responsible for defence against helminthic and arthropod infections and are also an essential component of allergic type reactions (Mosmann and Coffman, 1989). Mature Th2 cells preferentially secrete

a number of cytokines, including IL-10. The antibodies stimulated by this response, however, do not promote phagocytosis or activate complement efficiently.

Thus, the maturation of naïve $CD4^+$ T cells into the appropriate Th1 or Th2 phenotype is essential to launch an effective host response to an infection. As IFNγ is the signature cytokine for the $CD4^+$ Th-1 cytokine response and IL-10 is a Th2 cytokine, these data suggests that the both the occurrence of and outcome following severe sepsis may be linked to an imbalance in cytokine production in response to infection, with a dominant Th1 profile providing the optimal defence against infection.

Additionally, patients who developed severe sepsis had TNFα mRNA levels intermediate between that observed in the control and bacteraemic groups. This exaggerated TNFα response in patients with infection who did not develop critical illness may represent an appropriate and protective response. This concept, that certain pro-inflammatory cytokines are beneficial to patients with infection may account for the adverse outcome observed in prior studies of TNFα antagonists in patients with septic shock (Dhainaut et al., 1995; Fisher et al., 1996; Reinhart et al., 1996). Furthermore, such a protective role of TNFα in sepsis may in part account for the association with persistent shock and death that we observed in conjunction with lesser TNFα mRNA levels in ICU patients with severe sepsis.

As with TNFα, IFNγ possesses pro inflammatory properties. IFNγ is a pleiotrophic cytokine, which augments phagocytic bactericidal activity (Boehm et al., 1997), and consequently the lower IFNγ mRNA levels observed in patients with severe sepsis and persistent shock is not likely to be a beneficial response. In a similar fashion to the pattern of TNFα gene expression, the relatively greater levels of IFNγ mRNA observed in bacteraemic patients in comparison to the ICU patients may

represent an appropriate and beneficial Th1 cytokine response. This concept is consistent with animal models of sepsis, which confirms the importance of IFNγ in bacterial clearance (Deng et al., 2004; Ono et al., 2001; Pammit et al., 2004).

The discordant patterns of TNFα and IFNγ mRNA production observed between the ICU and the bacteraemic groups may be explained by the different cellular origins of these cytokines. TNFα is produced by a wide variety of cell types, but primarily by macrophages, whereas IFNγ is primarily produced by $CD4^{+}$ Th1 lymphocytes and NK cells (Boehm et al., 1997).

In humans, several case series document the beneficial use of exogenous IFNγ in leishmaniasis, leprosy and multi-drug resistant tuberculosis (Barral-Netto et al., 1999; Condos et al., 1997; Haas et al., 2002). The therapeutic use of IFNγ in patients with sepsis has been less extensively reported. To date, two studies failed to show any survival benefit from therapy with subcutaneous IFNγ in patient groups prone to develop sepsis (Polk et al., 1992; Wasserman et al., 1998). However, a case series of patients with severe sepsis coupled with documented monocyte suppression appeared to benefit from daily subcutaneous IFNγ (Docke et al., 1997). Similarly, trauma patients with diminished immune responsiveness, as measured by monocyte MHC class II receptor status, were less likely to develop nosocomial pneumonia when treated with inhaled IFNγ (Nakos et al., 2002).

The data presented in this study utilises the sensitivity of QRT-PCR as a method for evaluating cytokine gene expression. Prior investigations of cytokine responses to infection relied exclusively on ELISA based protein assays and, with the exception of IL-6 were either unable to detect pro-inflammatory cytokines in a significant proportion of patients, or to relate cytokine levels to clinical events (Bjerre et al., 2004; Damas et al., 1997; Goldie et al., 1995). However, a QRT-PCR based

mRNA assay provides uniquely sensitive *in vivo* information, not relying on *ex vivo* stimulation of cells to produce adequate amounts of material for detection which creates an artefactual setting. Therefore, QRT-PCR allows us to investigate absolute levels of gene expression *in vivo*, which can be used as an index of the adequacy of the cytokine response to infection.

4.6 Conclusion

These data demonstrate the utility of measuring cytokine mRNA by QRT-PCR in patients with infection. We show a marked association between the pattern of cytokine gene expression and the occurrence of severe sepsis, persistent shock and also with survival. Data are presented demonstrating a clear association between adverse outcome and an anti-inflammatory cytokine response. In effect, patients that tolerated infection with relative impunity showed a predominant Th1 response with over-expression of TNFα and IFNγ, and under-expression of IL-10 in comparison with the group that developed severe sepsis. Therefore, these data suggests that the occurrence of severe sepsis may be linked to an imbalance in cytokine production secondary to infection, with a dominant proinflammatory Th1 response providing the optimal defence against infection, associated with a reduced severity of disease and lesser mortality.

Chapter 5 - Results (3): Regulation of the Th1/Th2 cellular response following an infectious insult

5.1 Introduction

This section deals with the main putative regulators of the $CD4^+$ Th1 / Th2 cellular response and includes data on IL-4 and IL-12, cytokines that were originally thought to be the principal regulators of T cell maturation, and also on the more recently characterised cytokines IL-18, IL-23 and IL-27. Throughout the results section cytokine copy numbers are expressed as mRNA copy numbers standardised per 10 million β actin mRNA copies.

5.2 Comparisons between the three groups

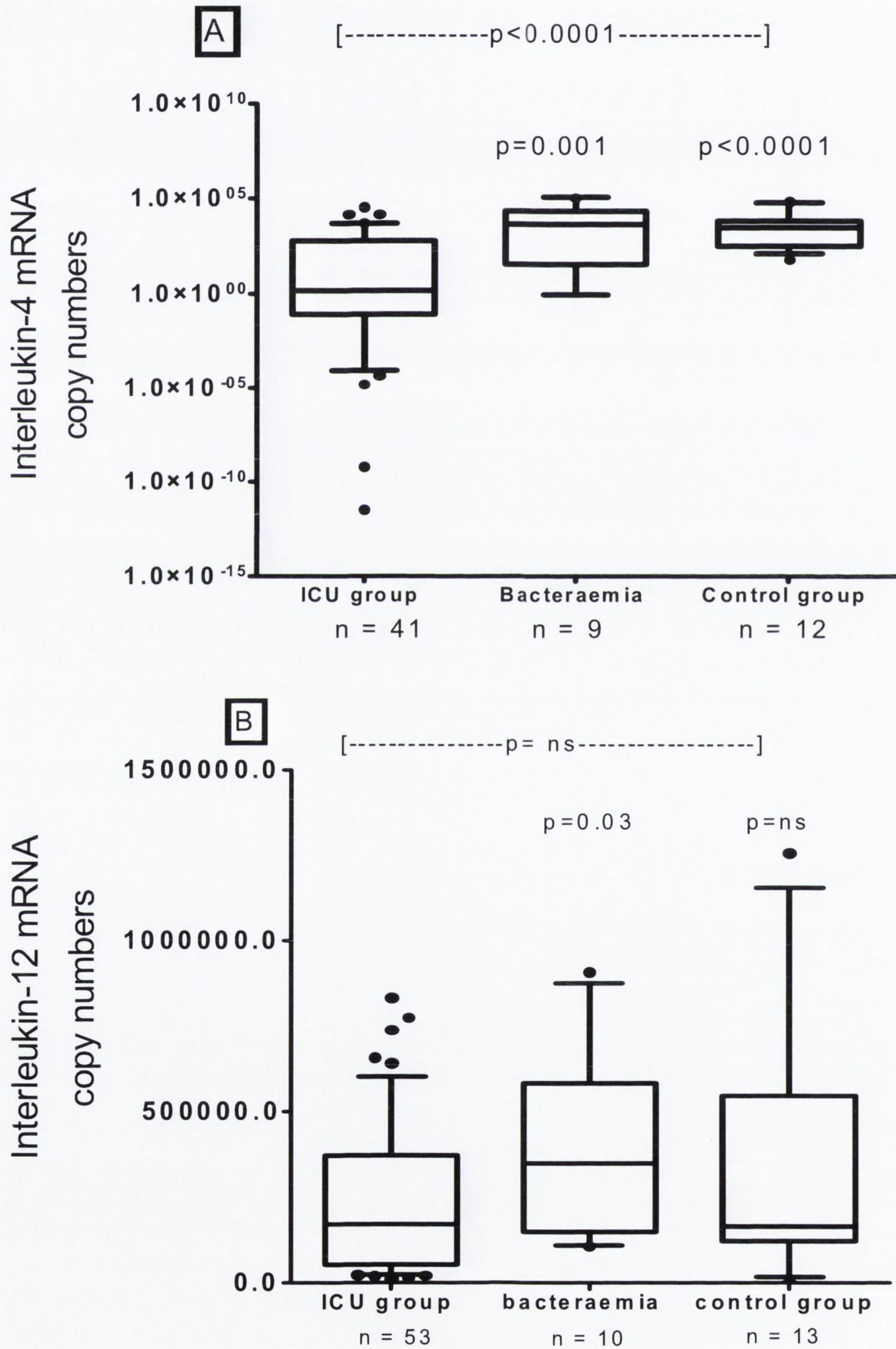
IL-4 mRNA levels in the ICU group on day 1 were lower than those of the control and bacteraemic groups ($p< 0.0001$, figure 5.1A). IL-12 mRNA levels in the ICU group on day 1 were less than those recorded in bacteraemic patients ($p=0.03$) but were similar to levels found in the control group (figure 5.1B).

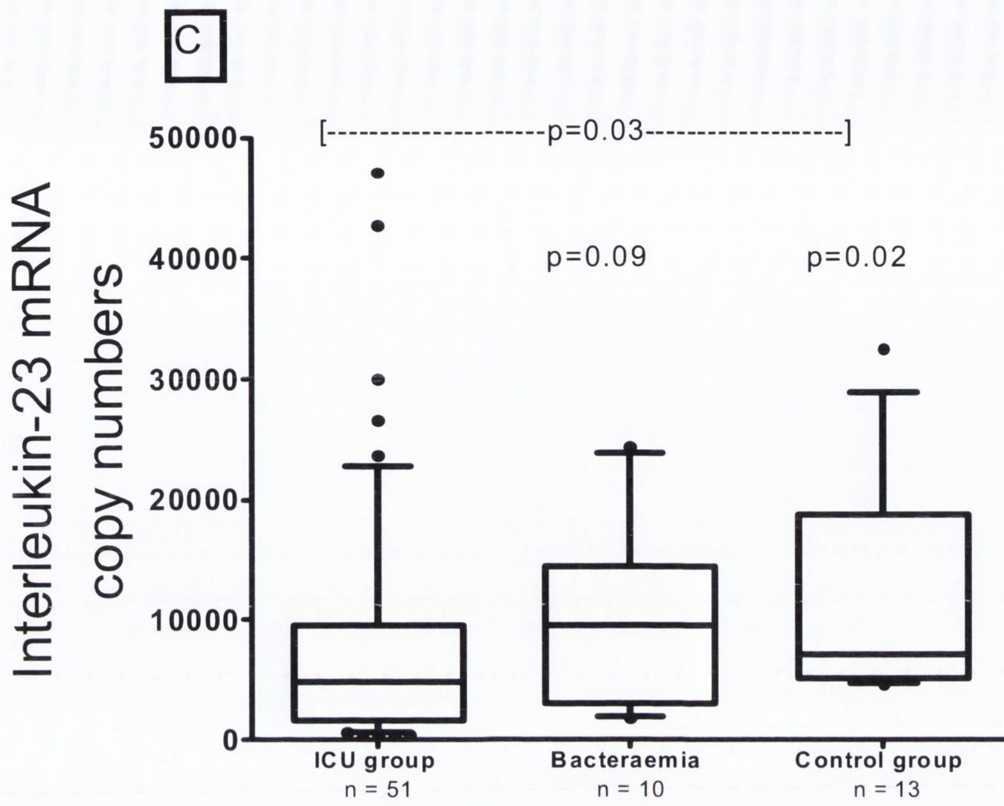
There was a difference in IL-23 mRNA expression when the 3 groups were analysed together ($p = 0.03$), with the ICU group demonstrating the lowest IL-23 mRNA levels (figure 5.1C). IL-27 mRNA levels also differed significantly between the three groups ($p = 0.003$). However, in this analysis the ICU group had the greatest levels followed by the bacteraemic group with the control group having the least amount of detectable IL-27 mRNA (figure 5.1D). The IL-27: IL-23 ratio demonstrated a similar pattern with the highest ratio in the ICU group ($p = 0.0003$, figure 5.1E). IL-18 mRNA levels did not distinguish between the three groups (figure 5.1F).

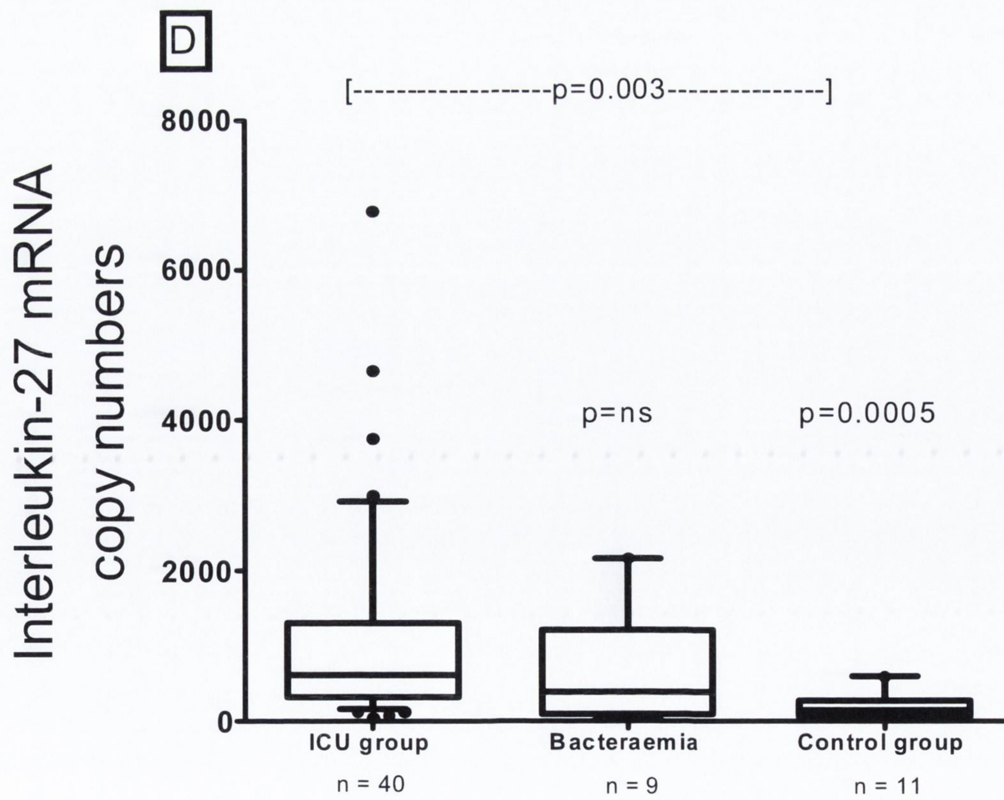
When the ICU group was compared with the combination of both the control and bacteraemia groups, significantly less IL-23 ($p = 0.008$) and greater IL-27 ($p = 0.003$) was detected in the ICU group in comparison with the combined group.

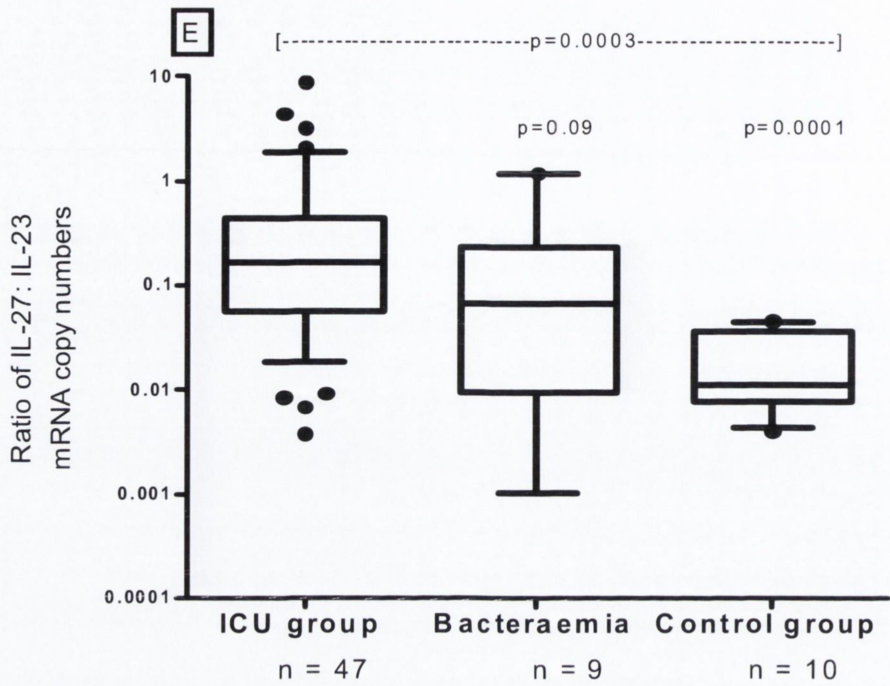
Similarly the IL-27: IL-23 ratio was greater in the ICU group compared with the combined group (p = 0.0002). There was no difference in IL-18 mRNA levels when the ICU group was compared with the combined group.

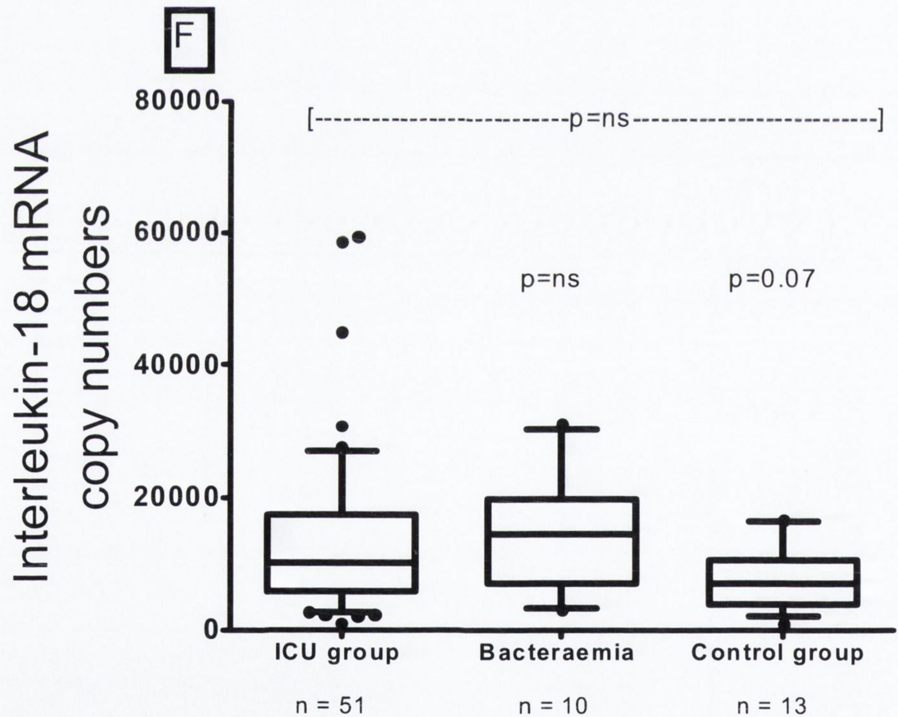
Figure 5.1




C
[-------------------p=0.03------------------]
p=0.09
p=0.02
Interleukin-23 mRNA copy numbers
50000
40000
30000
20000
10000
0
ICU group
n = 51
Bacteraemia
n = 10
Control group
n = 13


D
[-----------------p=0.003---------------]
p=ns
p=0.0005
Interleukin-27 mRNA copy numbers
8000
6000
4000
2000
0
ICU group
n = 40
Bacteraemia
n = 9
Control group
n = 11





Cytokine mRNA levels across the ICU group on day 1, the non-critically ill bacteraemia group and the control group. Values, represented as box plots, are median, quartiles 25% to 75% (box), and centiles 10% to 90% (whiskers). Individual p values are for comparison with the ICU group. The p value at the top of the figure represents a 3-group comparison.

5.3 ICU group

5.3.1 Description of the proximal cytokines on day 1

In the ICU group on day 1, median IL-4 mRNA levels were 11 (IQR 5-53). There was a non-significant trend for patients with lower IL-4 mRNA on day 1 to require inotropes (9 (4 – 25) Vs 27 (9 – 192), p=0.055). Whilst the SAPS2 score and IL-4 levels were inversely related (n= 42, spearman's rho 0.4, p= 0.009), there was no association between mRNA levels and other organ failure scores, lactate levels, extent of lung injury or type of infecting organism.

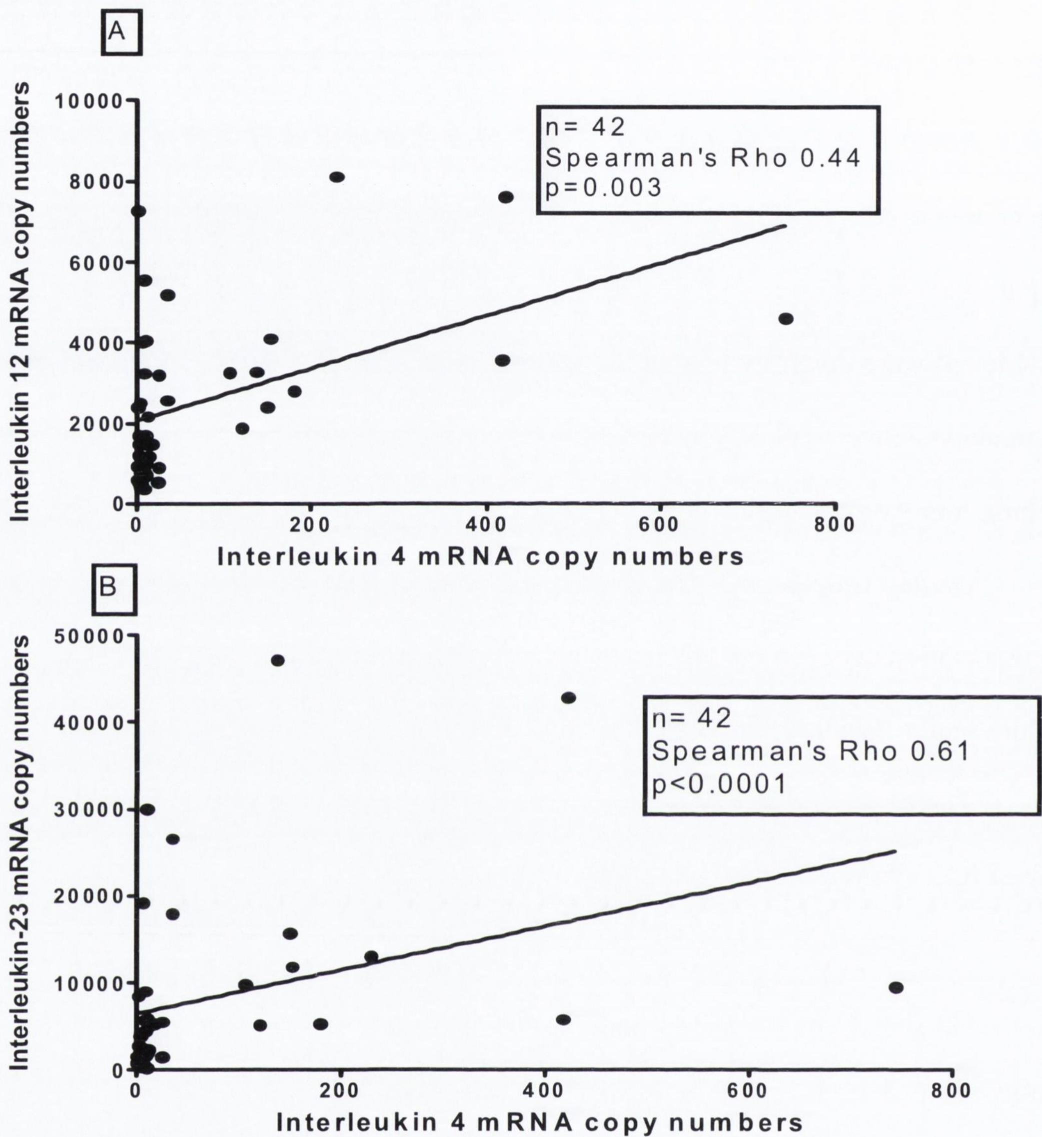
On day 1 median IL-12 mRNA levels were 2170 (IQR 923 – 4040). There was no association between mRNA levels and requirement for inotropes, any of the organ failure scores, lactate levels, extent of lung injury or type of infecting organism.

There was a direct correlation between IL-4 and IL-12 mRNA levels on the day of ICU admission (p= 0.003, figure 5.2A).

On day 1, in the ICU group, median IL-23 levels were 4767 (IQR 1561 – 9488), median IL-18 levels were 10224 (IQR 5824 – 17460), median IL-27 levels were 624 (IQR 322 – 1312) and the median IL-27: IL-23 ratio was 0.16 (IQR 0.06 – 1.03). We failed to observe a relationship between any of these cytokines and the requirement for inotropes, organ failure scores, extent of lung injury or the type of infecting organism.

However, there was a direct correlation between IL-4 and IL-23 mRNA on day 1 (p< 0.0001, figure 5.2B). No relationship was detected between IL-4 and either IL-27 or IL-18.

Figure 5.2



Correlations between the proximal cytokines. A. There was a direct correlation between IL-12 and IL-4 on day 1 of ICU stay (p=0.003). B. There was a direct correlation between IL-23 and IL-4 on day 1 of ICU stay (p<0.0001).

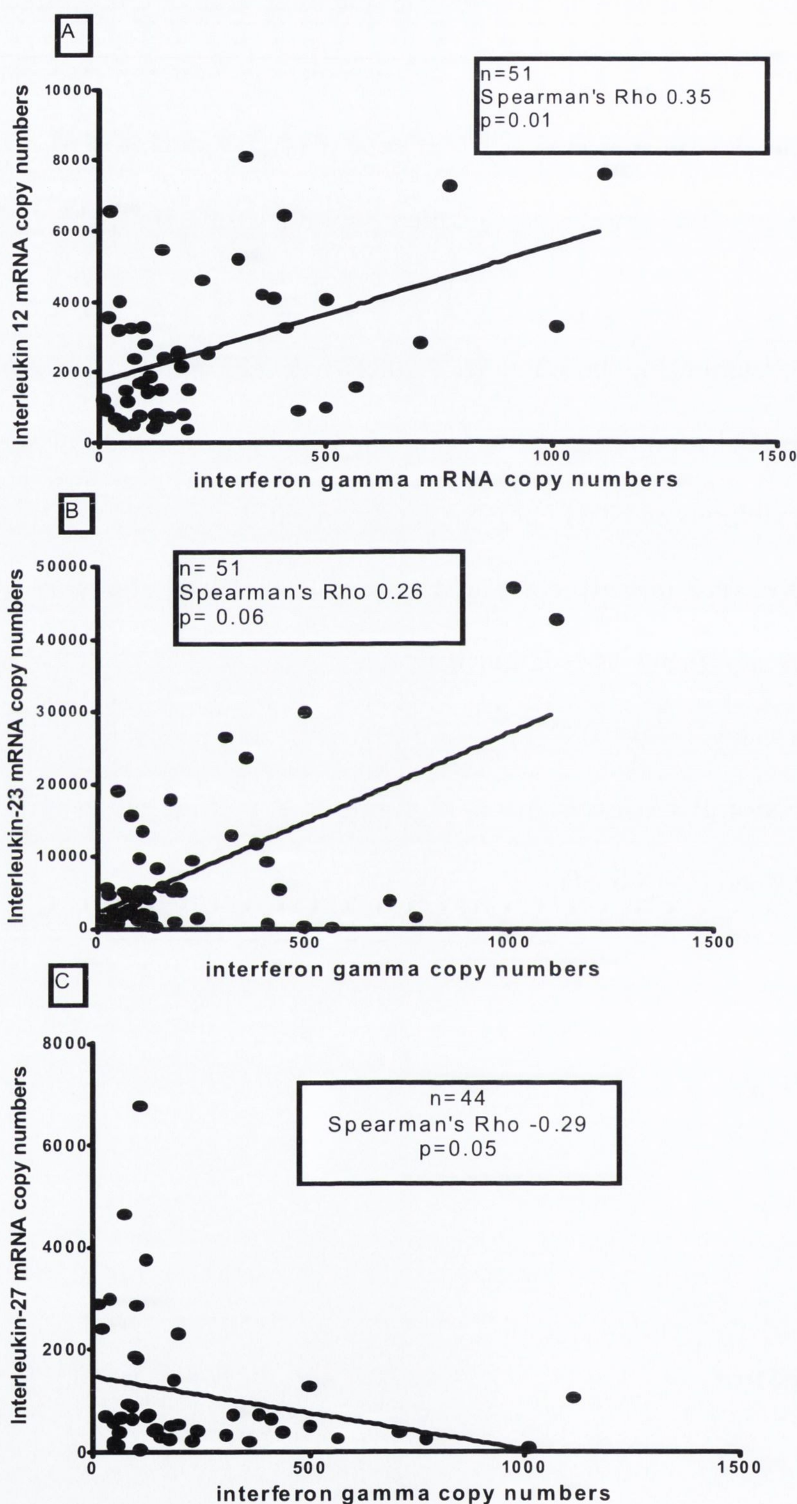
5.3.2 Relationship between the proximal and distal cytokines on day 1

Given the current information available on the regulation of the effector cytokines it is reasonable to examine for an association between the anti-inflammatory cytokine IL-10 and the Th2 regulator IL-4 and between the proinflammatory cytokines, TNFα and IFNγ and their putative regulators IL-12, IL-18 and IL-27.

There was no relationship between IL-4 mRNA levels and IL-10 mRNA levels on day 1 in the ICU group. Similarly, no relationship was observed between any of the regulatory cytokines and TNFα mRNA levels on day 1.

IL-12 and IFNγ were directly correlated on day 1 ($p = 0.01$, figure 5.3A). There was also a non-significant association between increased IL-23 mRNA and increased IFNγ mRNA levels ($p = 0.06$, figure 5.3B) and an inverse relationship between IL-27 and IFNγ mRNA levels ($p = 0.05$, figure 5.3C). No relationship was observed between IFNγ and IL-18 on day 1.

Figure 5.3



Correlation between the proximal and distal cytokines on day 1 of ICU stay. A. There was a direct relationship between IFNγ and IL-12 on day 1 of ICU stay (p=0.01). B. There was a borderline direct correlation between IFNγ and IL-23 on day 1 of ICU stay (p=0.06). C. There was a borderline inverse correlation between IFNγ and IL-27 on day 1 of ICU stay (p=0.05).

5.3.3 Description of the proximal cytokines on day 7

On day 7, in the ICU group, median IL-4 levels were 78 (IQR 26 – 294), median IL-12 levels were 4267 (IQR 2175 – 6886) and median IL-18 levels were 12841 (IQR 7025 – 19449). There was no association between these cytokine mRNA levels on day 7 and requirement for inotropes, organ failure scores, extent of lung injury, lactate levels or the type of infecting organism.

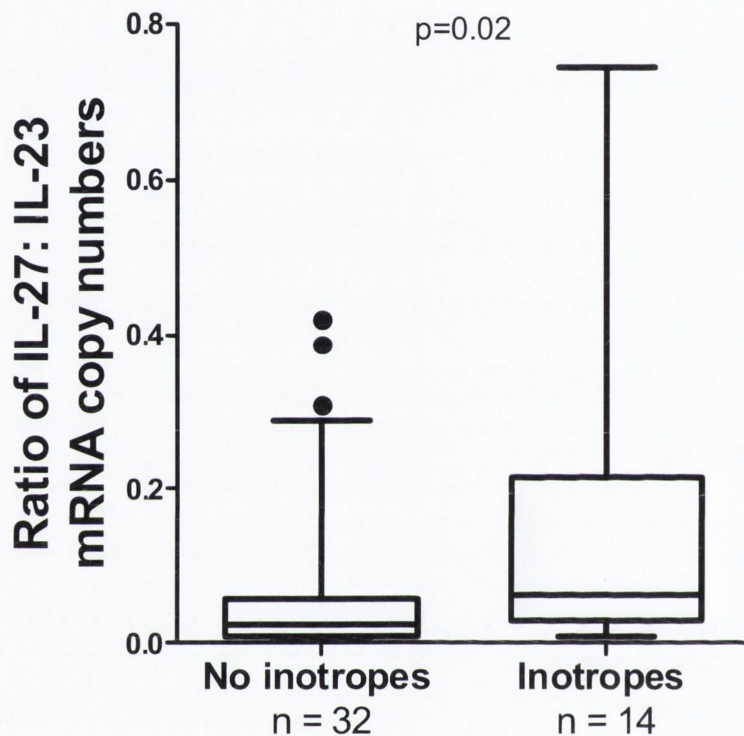
On day 7 median IL-23 levels were 9148 (IQR 4052 – 22275). IL-23 mRNA did not distinguish between patients with severe sepsis and those with septic shock. There was an inverse correlation between IL-23 mRNA and lactate levels (n=42, spearman's rho 0.4, p=0.008), SOFA score (n=49, spearman's rho 0.35, p=0.01) and MODS (n= 49, spearman's rho 0.46, p= 0.0009). There was a non-significant trend for higher IL-23 mRNA levels to be associated with higher PaO2 / FiO2 ratios (n = 42, spearman's rho 0.28, p= 0.06). There was no association between the type of infecting organism and IL-23 levels on day 7.

Median IL-27 levels were 279 (IQR 122 – 557) at this time point. There was a non-significant trend for higher IL-27 mRNA levels to be associated with the requirement for inotropes (214 (93 – 540) Vs 446 (196 – 918), p=0.07). However, there was no association between IL-27 mRNA on day and organ failure scores, lactate levels, extent of lung injury or type of infecting organism.

Higher IL-27: IL-23 ratios on day 7 were associated with the requirement for inotropes on day 7 of ICU stay (p=0.02, figure 5.4). The IL-27: IL-23 ratio also correlated with SOFA scores on day 7 (p = 0.01, figure 5.5A). There was an inverse correlation between IL-27: IL-23 ratio and PaO2/FiO2 ratios (n=39, spearman's rho 0.33, p=0.03) and higher lactates were associated with increasing IL-27: IL-23 ratios (p= 0.02, figure 5.5B).

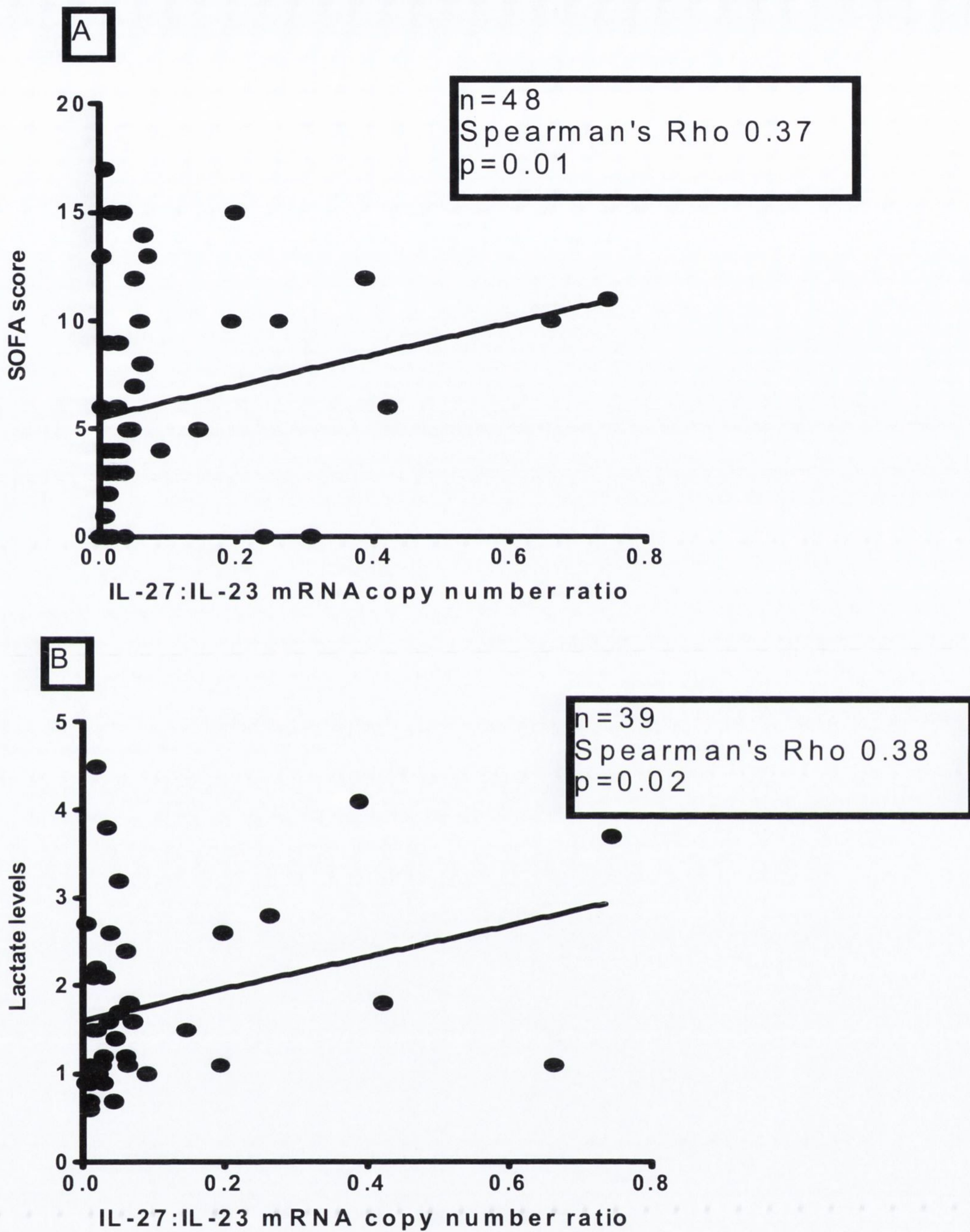
However, there was no observable relationship at this time point between IL-4 and any of the Th1 regulators studied; IL-12, IL-18, IL-23 or IL-27.

Figure 5.4



Inotrope requirements on day 7 of ICU stay. Values, represented as box plots, are median, quartiles 25% to 75% (box), and centiles 10% to 90% (whiskers).

Figure 5.5



Distal cytokines on day 7 and severity of illness. A. There was a direct correlation between the IL-27:IL-23 ratio and SOFA score on day 7 of ICU stay (p=0.01). B. There was a direct relation ship between IL-27:IL-23 ratio and lactate levels on day 7 of ICU stay (p=0.02). Lactate measured as mg/dL.

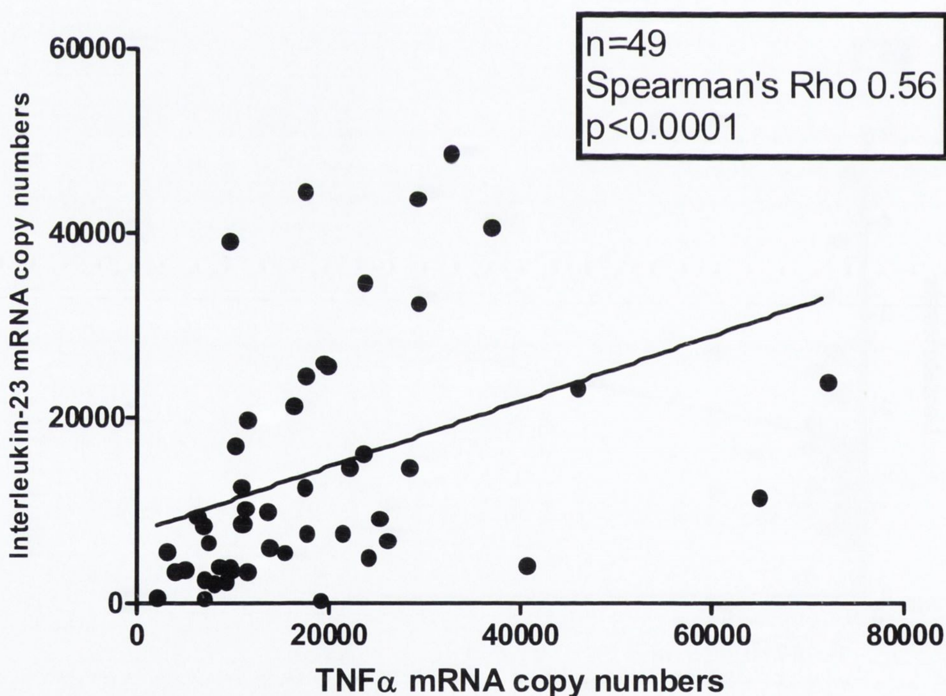
5.3.4 Relationship between the proximal and distal cytokines on day 7

Similar to our findings on day 1, IL-10 and IL-4 mRNA levels were unrelated on day 7 of ICU stay nor was there any relationship between IFNγ mRNA levels and the levels of IL-12, IL-18, IL-23 or IL-27 mRNA on day 7.

However, there was a significant correlation between IL-23 mRNA levels and TNFα mRNA levels ($p < 0.0001$, figure 5.6).

No relationship was detected between TNFα and IL-12, IL-18 or IL-27.

Figure 5.6



Correlation between proximal and distal cytokines on day 7 of ICU stay. There was a direct relationship between TNFα and IL-23 mRNA on day 7 of ICU stay ($p<0.0001$).

5.3.5 Change in mRNA levels over time

The ICU patients with RNA analysis available at both time points were assessed for a change in mRNA levels over time. In these patients there was no significant change in IL-18 or IL-27 mRNA levels over the seven days of critical illness, whereas IL-12, IL-4 and IL-23 mRNA levels increased (table 5.1).

Table 5.1: Changes in Cytokine mRNA levels over 7 days in ICU patients.

Cytokine	**N**	**Day 7 minus day 1 mRNA levels**	**p value**
IL-12	39	0.35 (-0.03 to 0.77)	0.001
IL-4	29	0.56 (0.2 to 1.07)	0.001
IL-23	38	0.38 (-0.04 to 0.79)	0.003
IL-18	39	0.15 (-0.43 to 0.43)	Ns
IL-27	34	-0.27 (-0.62 to 0.14)	Ns

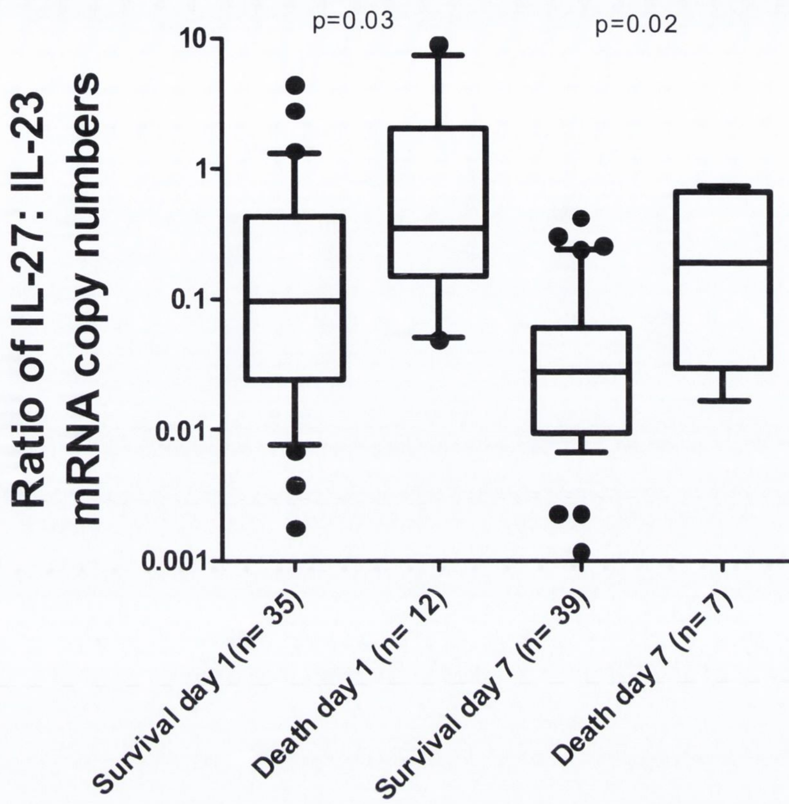
Results are expressed as differences in log mRNA copy numbers per 10^7 β actin mRNA copies when day 7 values are subtracted from day 1 values.
All values are expressed as median difference with interquartile range. Analysis is by Wilcoxon rank sign test.

5.3.6 Relationship to mortality

Seventeen of the 62 ICU patients (27%) died during the course of their ICU stay. On the first day of critical illness, from the 52 patients with mRNA available for analysis, 13 died prior to ICU discharge. There was a non-significant trend towards greater IL-23 mRNA levels ($p = 0.07$) and lesser IL-27 mRNA levels ($p = 0.08$) in survivors when compared with non-survivors. Higher IL-27:IL-23 ratios on day 1 of ICU ($p= 0.03$) were associated with an increased mortality (table 5.2, figure 5.7).

Eight of the 49 ICU patients, who had blood samples analysed for mRNA levels on the seventh day of critical illness, died prior to ICU discharge. These 8 non-survivors had lower IL-23 mRNA levels ($p = 0.03$) and increased IL-27:IL-23 ratios ($p = 0.02$) when compared to survivors (table 5.2, figure 5.7).

Figure 5.7



Risk of death in the ICU group and IL-27: IL-23 ratio. Values, represented as box plots, are median, quartiles 25% to 75% (box), and centiles 10% to 90% (whiskers). p values are for comparisons between survivors and non survivors.IL-27:IL-23 ratios were higher in non-survivors of sepsis on both day 1 (p=0.03) and on day 7 (p=0.02).

Table 5.2: Cytokine mRNA levels and outcome in the ICU group

	Survivors	Non-survivors	P value
N	45 (73%)	17 (27%)	
IL-12 day 1	2282 (917 – 4090)	1890 (993 – 3234)	ns
IL-12 day 7	4672 (2619 – 6886)	2420 (1588 – 6338)	ns
IL-4 day 1	11 (9 – 112)	5 (3 – 46)	ns
IL-4 day 7	78 (31 – 292)	179 (10 – 955)	ns
IL-23 day 1	5286 (1851 – 13179)	2121 (1305 – 5527)	0.07
IL-23 day 7	10191 (4782 – 24261)	5272 (995 – 9114)	0.03
IL-27 day 1	536 (241 – 972)	708 (428 – 2889)	0.08
IL-27 day 7	289 (107 – 542)	200 (144 – 1086)	ns
IL-18 day 1	10257 (6459 – 18550)	7758 (5346 – 14775)	ns
IL-18 day 7	11996 (6816 – 20740)	12866 (8729 –16724)	ns
IL-27:IL-23 day 1	0.1 (0.02 – 0.4)	0.35 (0.15 – 2.02)	0.03
IL27:IL-23 day 7	0.03 (0.01 – 0.06)	0.19 (0.03 – 0.66)	0.02

Results are expressed as mRNA copy numbers per 10 million β actin mRNA copies. All values are median and inter quartile range. ns, non significant.

5.4 Discussion

The previous chapter presents data suggesting that both the occurrence of, and the outcome following severe sepsis, may be linked to an imbalance in cytokine production in response to infection, with a dominant pro-inflammatory Th1 profile providing the optimal defence against infection. Consequently, of particular importance to the host's response to an invading pathogen is the induction of different elements of the $CD4^+$ T helper cell population. This distal cytokine response to infection that we have just described is regulated primarily by the induced $CD4^+$ T cell population, which in turn is determined primarily by the antagonistic interaction of the cytokines IL-12 and IL-4. These cytokines induce naïve $CD4^+$ T cells to differentiate phenotypically into Th1 or Th2 cells respectively (Swain et al., 1990; Wu et al., 1993) and were initially characterised as the dominant cytokines influencing naïve T cell differentiation. The selective differentiation of precursor $CD4^+$ T cells into effector Th1 and Th2 cells is established during the initial priming of these cells and whilst influenced by a number of extracellular factors, such as antigen load, mode of antigen presentation and the ligation of select co-stimulatory molecules, the most effective polarising factor is the cytokine milieu present during the Th cell differentiation process (Szabo et al., 2003).

We observed that the ICU group had a greater IL-12: IL-4 ratio than the bacteraemic group. This response appears appropriate and the net effect should have polarised $CD4^+$ T lymphocyte differentiation into a Th1 response, characterised by greater IFNγ production. However, IFNγ mRNA levels in patients with severe sepsis were lower than in control and bacteraemic patients. This data is clearly discordant given the role of IL-12 in stimulating IFNγ expression, and suggests that within the pathway of $CD4^+$ T cell differentiation, the molecular basis of the aberrant $CD4^+$ Th

cell polarisation observed in patients with severe sepsis is disconnected from the antagonistic interaction of IL-12 and IL-4.

The direct relationship observed between the Th2 polarising cytokine IL-4 and the Th1 cytokines IL-12 and IL-23 is interesting. This may reflect the activation of counter-regulatory mechanisms involving inflammatory and anti-inflammatory pathways in an attempt to limit any potentially pathogenic inflammation. There is evidence that many stimuli that increase pro-inflammatory mediators, particularly by activating NFκB also induce anti-inflammatory cytokines (Moore et al., 2001). However, this response was not observed in the effector cytokines in our ICU cohort. Additionally, this response is not observed later in the course of the disease and may possibly represent a feature of disease progression.

IL-12 is of particular interest in the elaboration of a cytokine mediated inflammatory response to sepsis, as this cytokine regulates production of IFNγ by cells of both the innate and adaptive immune systems (Boehm et al., 1997). Furthermore, IL-12 gene deletion experiments indicate that IL-12 has an important role in the clearance of pathogenic bacteria (Cooper et al., 2002).

Interestingly, IL-12 mRNA levels alone were not strongly related to the occurrence of severe sepsis, and not related to outcome in these ICU patients. This is surprising when one considers the importance of IL-12 in T cell differentiation. IL-12 is secreted predominantly by antigen presenting cells and acts on naïve $CD4^+$ cells, via the transcription factor STAT 4, to promote differentiation into a Th1, IFNγ producing, cell-type (Murphy et al., 2000). In animal models this effect is quite marked, with STAT 4 knockout mice displaying a deficit in bacterial clearance, which is partially reversed by exogenous IFNγ (Deng et al., 2004). Curiously, the impaired immune response observed in this STAT 4 knockout model appears to be qualitative

rather than quantitative, as the mice can mount an adequate leukocyte response to infection, but with inadequate bacterial clearance.

Furthermore, IL-12 therapy increases survival in animal models of sepsis (Ono et al., 2001; Pammit et al., 2004). A similar phenomenon has been observed in humans with sepsis, where lesser production of IL-12, specifically in response to endotoxin, is predictive of death from severe sepsis after major surgery (Weighardt et al., 2002). However, the lack of variability in IL-12 levels between groups observed in this study suggests that, within the cytokine cascade, the basis for an inadequate Th1 response lies somewhere between IL-12 and IFNγ.

IL-12 is a heterodimeric protein composed of two subunits, p40 and p35, encoded by unrelated genes (Gately et al., 1998). Neither subunit has biological activity alone, although a p40 homodimer may act as an IL-12 antagonist (Gately et al., 1998). Furthermore, IL-12 production by monocytes results in a 500-fold excess of p40 relative to the active heterodimer (D'Andrea et al., 1992). Approximately 20-40% of the p40 in the serum of normal and endotoxin-treated mice is in the form of the homodimer (Heinzel et al., 1997). In addition, the p40 subunit may lack sensitivity as it forms a common component of other heterodimeric cytokines such as IL-23 (Vanden Eijnden et al., 2005). As a consequence of this we chose to measure the p35 subunit as an index of the IL-12 heterodimer activity.

Similarly, the exaggerated Th2 response, in terms of IL-10 production, observed in the ICU cohort was not associated with increased production of its' primary regulator, IL-4. This is again surprising given the central role played by IL-4 in the differentiation of a naïve $CD4^+$ precursor cell into a mature Th2 IL-10 producer. It is plausible, however that this Th2 response was driven by other factors, functioning independently of IL-4 production.

Therefore, although we have demonstrated that both the mild and severe responses to infection can be predicted by the induced Th cell population, these responses appear independent of the main putative regulators of Th1/Th2 development, namely IL-4 and IL-12.

Whereas IL-4 remains the pre-eminent cytokine for inducing Th2 differentiation, it appears that the critical importance of the Th1 immune response has led to the evolution of multiple mechanisms for the induction and regulation of Th1 cells involving a plethora of more recently discovered cytokines. These include IL-18, originally termed IFNγ inducing factor, and two cytokines sharing subunits with IL-12; IL-23 and IL-27. In addition, IL-23 and IL-27 are pleotropic and are also involved in the regulation of another effector population of $CD4^+$ T cells; the IL-17 producing Th17 lineage of T cells, purported to be of critical importance in driving an autoimmune inflammatory response and also in host protection against bacterial infection (Hunter, 2005; Ye et al., 2001).

We have found that the occurrence of severe sepsis and the mortality associated with the septic insult was linked to a distinct pattern of regulatory cytokine gene expression, specifically excess IL-27 and lesser IL-23 mRNA levels. We failed to demonstrate a relationship between IL-18 mRNA and outcome or severity of illness.

Early work suggested that IL-23, which is produced by a wide range of cells, including dendritic cells, macrophages and mature T cells, exclusively acted with IL-12 to enhance IFNγ production (Szabo et al., 2003). More recently, it is accepted that IL-23 plays an important role in promoting inflammation through production of the IL-17 family of cytokines from a novel subset of $CD4^+$ T cells, termed Th17 cells (Hunter, 2005). The Th17 lineage of T cells have been purported to be of critical

importance both in driving a variety of autoimmune inflammatory responses, including inflammatory bowel disease and animal models of collagen induced arthritis and experimental autoimmune encephalitis, and also in host protection against bacterial infection (Chen et al., 2006; Colgan and Rothman, 2006; Hunter, 2005; Ye et al., 2001; Yen et al., 2006). Furthermore, while the interaction between the T cell subsets is complex, gene deletion studies indicate that IL-12 preferentially induces IFNγ production whilst IL-23 preferentially induces IL-17 production (Happel et al., 2005).

Interestingly, the IL-23 receptor is not present on naïve T cells but is detectable on memory $CD4^+$ T cells which suggests that IL-23 may be preferentially involved in sustaining inflammation in the later stages of a response rather than initiating a pro inflammatory response from naïve $CD4^+$ T cells (Parham et al., 2002). It appears that IL-23 is necessary for the permanent differentiation of the Th17 lineage thus promoting sustained IL-17 production with potentially profound effects on inflammation and immune function (Veldhoen et al., 2006). Additionally, by acting directly on macrophages to increase their production of TNFα, IL-23 has a role as an effector cytokine in innate immunity and, in a similar fashion to IL-12, provides an important link between innate and adaptive immunity (Uhlig et al., 2006).

IL-27 is a cytokine, structurally related to IL-12, whose definitive function has proved more elusive. Originally reported to promote the differentiation of Th1 cells from naïve human $CD4^+$ T cells by acting synergistically with IL-12 (Pflanz et al., 2002), subsequent data indicated a role in suppression of T cell responses (Hunter, 2005). Recently, it has been demonstrated that IL-27 can repress the pro-inflammatory $CD4^+$ Th17 response *in vivo* (Batten et al., 2006; Stumhofer et al., 2006), thereby acting in an antagonistic fashion to IL-23. Additionally, using a murine

gene knockout model, investigators have demonstrated that deficient IL-27 was associated with resistance to CLP-induced septic peritonitis (Wirtz et al., 2006). Data are presented suggesting a direct link between IL-27 and suppression of endotoxin-induced production of bactericidal products by macrophages whilst IL-27 blocking protein improved survival after CPL. Therefore, IL-27 may be a critical negative regulator of the immune response in sepsis.

Our data also supports opposing actions for IL-23 and IL-27. It is plausible that an imbalance between IL-23 and IL-27 gene expression in patients with infection contributes to an excess mortality by repressing protective inflammatory bactericidal responses dependant on either a $CD4^{+}$ Th1 or a Th17 cell response pattern. There are no prior studies of mRNA levels for these cytokines in humans with sepsis, so although we have demonstrated an association between reduced IL-23 mRNA and adverse outcome with infection, it is not possible to determine directly from our data whether this link is reactive or causal. However in animal models of sepsis, gene deletion studies indicate that the IL-23 / IL-17 pathway is protective (Happel et al., 2005; Happel et al., 2003), and it is plausible that the pathophysiology is similar in humans.

We observed that the effects of IL-23 and IL-27 were present both at the onset of sepsis and also in late sepsis, where a strong association between IL-23 and TNFα mRNA levels was apparent. We have previously demonstrated, in chapter 4, a linkage between deficient TNFα mRNA production in late sepsis and adverse outcome in this study population. Taken together, these data suggest a deficient protective immune response in late sepsis. Interestingly, as TNFα production may be regulated by IL-17 (Hunter, 2005; Lubberts et al., 2005), and IL-23 is a positive regulator of the $CD4^{+}$ Th17 phenotype, the linkage observed between IL-23 and TNFα in late sepsis,

suggests that a component of this immune deficiency may encompass the $CD4^{+}$ Th17 response.

Although the individual components of the innate and adaptive immune response to infection are well characterised, along with extensive study of the interaction of cellular and humoral components of the immune response, a clear understanding of the pathophysiology of severe sepsis and septic shock has to date proved elusive (Cohen, 2002). As IL-23 and IL-27 regulate cytokine production by differentiated $CD4^{+}$ cells the data presented in this study illustrates the importance of adaptive immunity in both the onset and resolution of severe sepsis in immune competent adults. This evidence adds to the current available data supporting a vigorous pro-inflammatory response as being of paramount importance in containing an infectious insult (Docke et al., 1997; Nakos et al., 2002; O'Dwyer M et al., 2006; Pachot et al., 2005a). As such, any benefit observed through antagonism of its effects in animal models is unlikely to translate to an improved outcome in a clinical scenario.

Genetic studies have also highlighted the potential importance of these genes in the human response to infection (Filipe-Santos et al., 2006). Disease causing mutations in p40 and in the shared receptor subunit of IL-12 and IL-23, IL-12Rβ1, were originally attributed to resultant alterations in the IL-12 / IFNγ response to infection (Altare et al., 1998a; Altare et al., 1998b; de Jong et al., 1998). Whilst it is currently appreciated that these mutations must also affect IL-23 dependant pathways (Fieschi et al., 2004; Filipe-Santos et al., 2006) the relative contributions of IL-12 and IL-23 has not been defined. It is interesting that, to date, no p35 or IL-12Rβ2 deficient patients have been identified. This may reflect the need for both the IL-12 and IL-23 genes to function in unison to mediate a competent immune system. The data we

present represent the first report confirming the potential importance of IL-23 gene expression in human sepsis *in vivo*.

Although the importance of IL-23 as a mediator of pathogenic inflammation in inflammatory bowel disease (IBD) is now appreciated it has been suggested that IL-23 mediates only the local inflammatory processes whereas IL-12 is of paramount importance to the systemic inflammatory response (Hue et al., 2006; Uhlig et al., 2006). These investigators propose that IL-23, acting via IL-17, promotes stromal, epithelial and endothelial cells and a subset of monocytes to produce a combination of cytokines which in turn lead to the recruitment of neutrophils to the site of infection or injury. In mice, colitis was completely dependant on IL-23, but not IL-12, whereas a p19 antibody completely inhibited the mucosal immunopathlogy but not the systemic immune pathology (Uhlig et al., 2006). However, we have demonstrated that systemic IL-23 mRNA levels accurately differentiates between groups based on the severity of response to a septic insult. IL-12, when measured similarly, did not differentiate between these groups.

Previously, IL-18 has been implicated as being causative in propagating sepsis induced organ failure by virtue of propagating an excessively vigorous pro-inflammatory response (Tschoeke et al., 2006). In contrast to this, we have previously described the benefits of a pro-inflammatory response in patients with sepsis (O'Dwyer M et al., 2006). Additionally, the current study demonstrates that IL-18 mRNA levels do not differentiate between critically ill infected patients and patients that tolerate infection with relative impunity and levels do not relate to severity of illness in the ICU population. Recent evidence from animal models supports this view (Moreno et al., 2006). As such, the role of IL-18 in human sepsis remains unclear.

5.5 Conclusion

This data adds substantially to the evidence in the previous chapter supporting the concept that a vigorous pro-inflammatory response is of paramount importance in containing an infectious insult (O'Dwyer M et al., 2006). As such, any benefit observed through antagonism of its effects in animal models is unlikely to translate to an improved outcome in a clinical scenario. Contrary to evidence from *in vitro* data (Hsieh et al., 1993) our data shows no strong evidence that IL-12 plays a dominant role in the development of a Th1 inflammatory response *in vivo*. Other cytokines are likely to play a central role with IL-23 and IL-27 likely to be critical importance. From these data IL-23 appears to be involved in the propagation of the immune response whilst IL-27 is likely to act as a suppressor of inflammation. The relative importance of the Th1 and Th17 pathways remains to be fully elucidated.

We suggest that, based on these data, IL-18 antagonists are unlikely to prove of benefit in a clinical scenario. However, up regulation of the Th1 or Th17 immune response with IL-23 or with IL-27 antagonists in a timely and targeted fashion warrants further research.

Chapter 6 - Results (4): Analysis of IκBL mRNA levels in response to an infectious insult

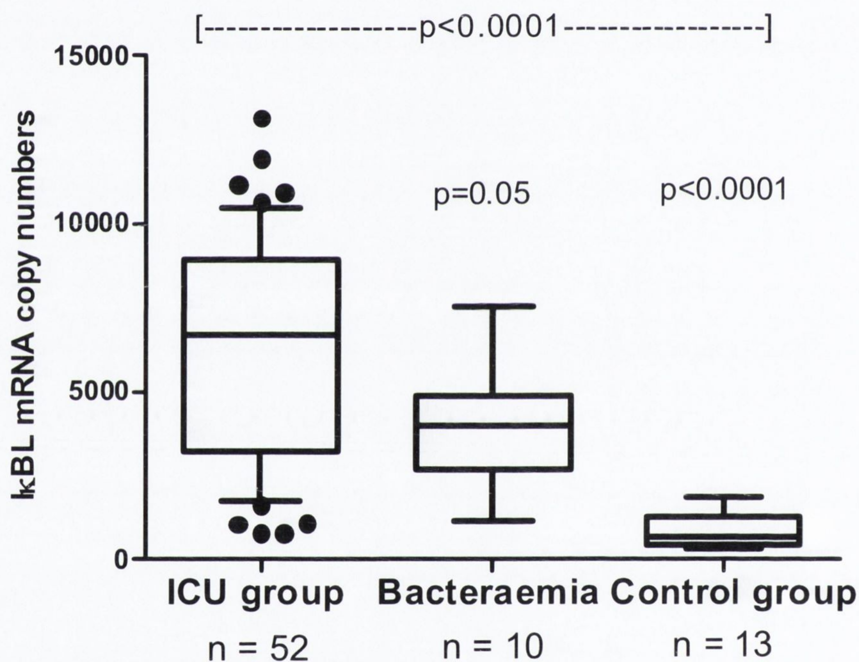
6.1 Introduction

IκBL is located telomeric of the TNF cluster in the central MHC on chromosome 6 (Albertella and Campbell, 1994). The deduced amino acid sequence of the IκBL gene shows that it comprises sequences that are known to be involved in protein-protein interactions and are also found in the IκB family of proteins allowing them to bind and thus regulate the NFκB/Rel family of transcription factors (Price et al., 2003). These and other observations suggest that IκBL may be an atypical member of this family and, although speculative, it is proposed that IKBL may function in a similar fashion as the IκB family.

6.2 Three group comparison for IκBL mRNA levels

IκBL mRNA levels distinguished between the ICU group on day 1, the bacteraemic group and the control group ($p<0.0001$) with IκBL mRNA most abundant in the sepsis group, intermediate in the bacteraemia group and lowest in the control group (figure 6.1).

Figure 6.1



IκBL mRNA levels across the ICU group on day 1, the non-critically ill bacteraemia group and the control group. Values, represented as box plots, are median, quartiles 25% to 75% (box), and centiles 10% to 90% (whiskers). Individual p values are for comparison with the ICU group. The p value at the top of the figure represents a 3-group comparison.

6.3 ICU group

6.3.1 Description of IκBL levels in the ICU group

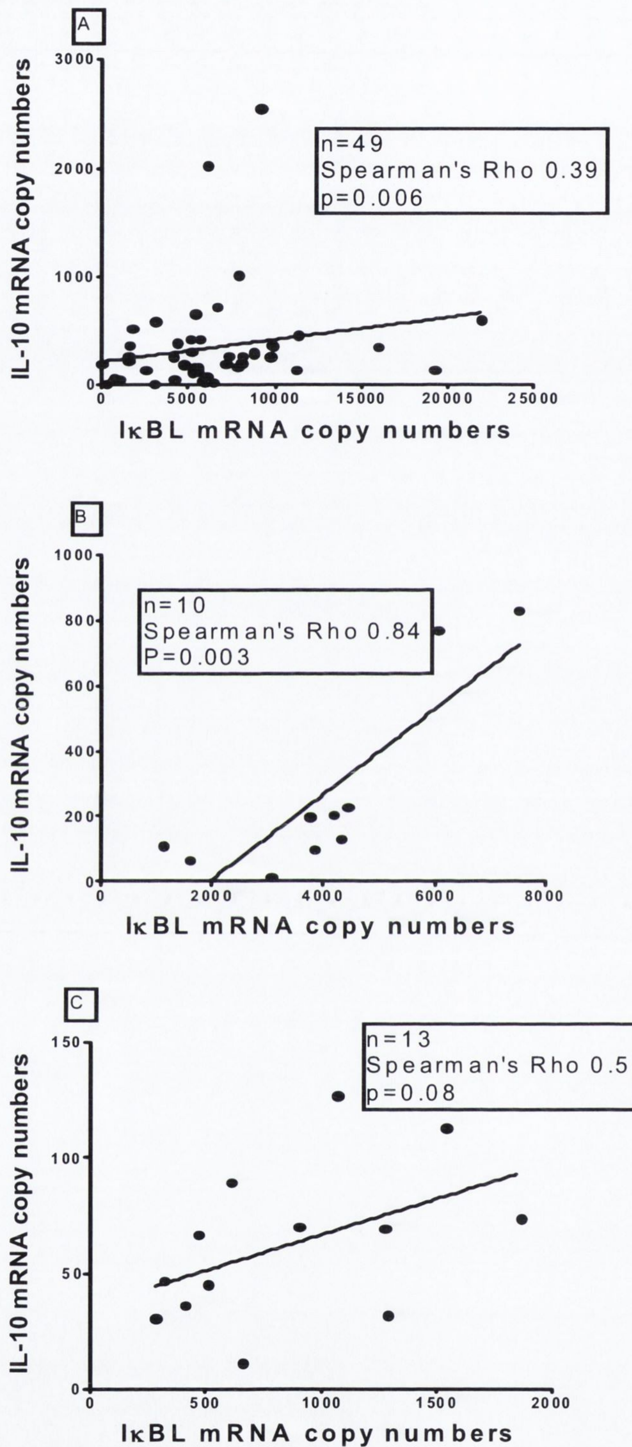
On day 1 in the ICU group IκBL mRNA was available for analysis in 52 patients. Median IκBL mRNA levels were 6670 (IQR 3226 – 8944). On day 7 IκBL mRNA was available for analysis in 49 ICU patients. Median IκBL mRNA levels were 5603 (IQR 2799 – 8105). IκBL data were available for 39 ICU patients at both time points. The difference between detectable levels on day 1 and day 7 was non significant ($p=0.7$). On both day 1 and on day 7 IκBL mRNA levels were unrelated to the requirement for inotropes, severity of organ failure, degree of acidosis or the type of infecting organism.

There was no relationship detected between IκBL mRNA levels and outcome in the ICU group at either time point. Similarly, there was no relationship between the extremes of IκBL (highest and lowest quartile) and outcome at either time point.

6.3.2 Relationship between IκBL mRNA and IL-10 mRNA levels

There was no association between IκBL mRNA levels and any of the previously characterised cytokines on day 1 in the ICU group. However, on day 7, in the ICU group, there was a direct correlation between IκBL mRNA and IL-10 mRNA ($p=0.006$, figure 6.2A). IκBL and IL-10 levels were also directly correlated in the bacteraemia group ($p=0.003$, figure 6.2B). In the control group there was a non-significant trend for increasing IκBL levels to be associated with increasing IL-10 levels ($p=0.08$, figure 6.2C).

Figure 6.2



Correlation between IL-10 and IκBL mRNA. A There was a direct correlation between IκBL and IL-10 mRNA on day 7 of ICU stay (p=0.006). B There was a direct correlation between IκBL and IL-10 mRNA in the non critically ill bacteraemia group (p=0.003). C There was a non significant relationship between increasing IκBL and increasing IL-10 mRNA in the control group (p=0.08).

6.3.3 Influence of the –62 IκBL polymorphism on gene expression

The distribution of IκBL –62 (A/T) alleles conformed to a Hardy-Weinberg equilibrium. At position –62 in the IκBL promoter 25 patients (41%) were A homozygotes, 10 patients (17%) were T homozygotes and 25 patients (42%) were heterozygotes. No association was detected between IκBL genotype and IκBL mRNA levels on either day 1 or day 7. Similarly, the extremes of production (highest and lowest quartiles) were not associated with any of the IκBL genotypes assessed.

6.4 Discussion

We have demonstrated that IκBL mRNA levels rise in response to an infectious insult and that the extent of that increase is related to the severity of the infectious insult. This is reflected in the excess of detectable IκBL mRNA in the ICU group in comparison to the bacteraemic group. Additionally, we have shown that IκBL and IL-10 mRNA levels rise in tandem in patients with infection raising the possibility of coordinated regulation or even a functional interaction between IL-10 and IκBL *in vivo*.

NFκB is a nuclear factor that stimulates the transcription of many pro-inflammatory cytokines by binding to specific sites in the promoter regions of these genes. NFκB is regulated by a number of proteins seemingly specialized in this task: the IκB protein family. IκBα is the archetypal member of this family and binds to NκFB, thereby masking the NFκB nuclear localization signal, preventing its' translocation to the nucleus and subsequent enhancement of gene expression. Phosphorylation of IκB proteins by the enzyme IκK allows the IκB proteins to be ubiquitinated and degraded, thus releasing NFκB (Wilson et al., 2005). Other members of the family have different modes of regulation; for example IκBz regulates NFκB even though it is thought to be located exclusively in the nucleus (Yamazaki et al., 2001).

The IκBL gene encodes for a 381 amino acid protein and contains ankyrin motifs similar to those of the IκB family of proteins. As IκBL shares homology with other members of the IκB family, it has been suggested that IκBL may interact with NFκB, in a similar fashion to IκBα, and thus may regulate cytokine production (Lin et al., 2006). Evidence from this laboratory shows that IκBL inhibits NFκB activity (Mankan et al, unpublished). Therefore, given this potential role, IκBL is a candidate

gene in the susceptibility to, and outcome from, sepsis. Furthermore, the genomic location of IκBL, at the telomeric end of the MHC on chromosome 6p21.3, surrounded by genes involved in inflammation such as TNFα, LTA, LTB and BAT1, further point to a potential role for IκBL in the regulation of inflammation.

If IκBL does indeed inhibit proinflammatory transcription factors such as NFκB, then a rise in levels following an infectious insult is likely to be a counter-productive host response, as increased IκBL levels would in this scenario limit defense mechanisms against infection. The distinct differences observed between groups in IκBL mRNA at the onset of the infectious insult mirror the pattern observed at the level of the distal effector cytokines. These data suggest that a suboptimal inflammatory response leads to an increase in the severity of illness in response to infection. Increased IκBL levels may be characteristic of this suboptimal inflammatory phenotype. Given the findings reported in the literature, and unpublished results from this laboratory, it is plausible that increased levels of IκBL in the ICU group in comparison to the bacteraemic and control groups may impair NFκB's ability to activate proinflammatory genes. The lesser amounts of IκBL observed in the bacteraemic group may contribute to a more effective immune response to infection, and thus allow these patients to tolerate infection with relative impunity by preserving, to a relatively greater extent, host bactericidal functions.

IL-10, a potent anti inflammatory cytokine, exerts it's anti inflammatory properties in a variety of ways, including activation of the genes SOCS-1 and SOCS-3, which inhibit pro-inflammatory cytokine signaling pathways, and also through stabilisation of the NFκB-IκB complex (Pestka et al., 2004). IL-10 inhibits the phosphorylation of IκK which is necessary for its activation, thereby inhibiting the proteolysis of IκBα and the release of NFκB (Schottelius et al., 1999). Therefore, an

important facet of IL-10's anti-inflammatory activities of IL-10 is to negatively regulate NFκB, thereby preventing its activation in response to infection or other stimuli.

There are scant data on the mode of action of IκBL in sepsis at present. We have described an association between IκBL and IL-10 mRNA in late sepsis which may be considered unsurprising viewed in the light of current data on the interactions between IκBα (and by extention, NFκB) and IL-10. Many studies have demonstrated that IL-10 inhibits the activity of NFκB in macrophages and T cells *in vitro* (Sica et al., 2000; Wang et al., 1995), whilst deficiencies in IL-10 production have been implicated in the propagation of prolonged and excessive inflammatory responses mediated via NFκB (Saadane et al., 2005). Interestingly, the proposed inhibition may be reciprocal, as a dominant negative IκB, which provides a highly effective biological block to NFκB activation and translocation resulted in unrestrained IL-10 synthesis with little or no synthesis of pro-inflammatory cytokines following exposure to LPS (Wilson et al., 2005).

We may speculate, therefore, that the increased levels of IL-10 observed in the ICU patients, in contrast to the bacteraemic group, may specifically interfere with any potential NFκB-IκBL interaction leading to reduced NFκB induced transcription of genes central to the hosts bactericidal activity. Alternatively, the raised IκBL levels observed may be a primary phenomenon leading in turn to enhanced IL-10 production thus inducing an anti-inflammatory phenotype in a patients with an active infection. Either of these scenarios may lead to the host possessing less than adequate bactericidal properties with a subsequent detrimental outcome. It may be useful in future studies to quantify NFκB activity along with a measurement of IκBL gene expression in order to confirm the mechanism of action.

A T/A polymorphism exists in the promoter region of IκBL at position –62. Polymorphisms at this site have been shown to disrupt the binding motif for the transcriptional factors δEF1, USF1 and E47 (Lin et al., 2006). We failed to show an association between the –62 SNP and IκBL mRNA levels or any clinical correlates. Previously, transfection experiments with luciferase reporter constructs carrying reporter alleles of IκBL showed an effect of IκBL –62 alleles on expression (Boodhoo et al., 2004) which have been confirmed in this laboratory (Caraher et al, unpublished). Additionally, the –62 SNP has been associated with susceptibility to numerous inflammatory disorders such as rheumatoid arthritis, ulcerative colitis and diabetes (de la Concha et al., 2000; Lin et al., 2006; Yamashita et al., 2004). Unfortunately, we do not have large enough groups to assess the influence of the –62 SNP on the susceptibility to sepsis. Genotype frequencies in control groups differ between populations with the frequencies of A homozygotes ranging from 33% to 10% in Asian control populations (Lin et al., 2006; Yamashita et al., 2004). There are no prior data on this SNP in a human septic setting. As the ICU cohort is relatively small the effect of different alleles on IκBL levels in sepsis would have to be very strong in order to be detected. Therefore, to definitively assess the association of this allele with outcome from sepsis, severity of disease, or its functionality *in vivo*, it will likely be necessary to study a much larger and thus statistically more powerful, cohort of patients.

6.5 Conclusion

We have described how IκBL mRNA levels may be used to discriminate between differing responses to a septic insult. Current evidence points to an interaction with NFκB, with increased levels of IκBL ultimately leading to a dampened bactericidal response. Whether this is a primary phenomenon or reactive to increased IL-10 levels remains unclear from the data presented here. However, the data presented is of importance as it provides a plausible mechanistic link between alterations in cytokine gene expression, subsequent alterations in extracellular cytokine levels and their intracellular effects. It is these intracellular effects that may determine how a host responds to an infectious insult. Further research may lead to greater understanding as to mechanism of action of increased IκBL levels and the position of IL-10 in the cascade. This is of obvious importance as immunomodulation therapy involving IL-10 may ultimately affect NFκB activity via a mechanism involving IκBL, thereby potentially affecting bactericidal properties in an infected host.

Chapter 7 - Results (5): Analysis of protein levels in the ICU and control groups

7.1 Introduction

Asymmetrical dimethyl arginine (ADMA) is a naturally occurring non-selective inhibitor of NOS, derived from protein catabolism, and is metabolised to citrulline by dimethylarginine dimethylaminohydrolase (DDAH) (Tran et al., 2003).

7.2 ADMA levels

7.2.1 Description of ADMA levels in the ICU and control groups

In total 47 ICU patients and 10 healthy controls had serum analysed for the presence of protein product. Samples were available for analysis from 40 ICU patients on day 1 and from 35 ICU patients on day 7. Twenty-eight patients had blood samples available for analysis at both time points. Fourteen (30%) patients died prior to ICU discharge. Demographic data, clinical details, and pertinent laboratory results for patients are detailed in Table 7.1.

On day one, 31 of the sampled ICU patients (66%) required an infusion of inotropes to maintain adequate arterial blood pressure. ADMA levels ($p = 0.001$, figure 7.1), lactate levels ($p=0.018$) and organ failure scores ($p=0.003$) were higher in the group requiring inotropes (table 7.2). Patients in this group on day 1 were also more likely to be non-survivors ($p=0.01$, table 7.2).

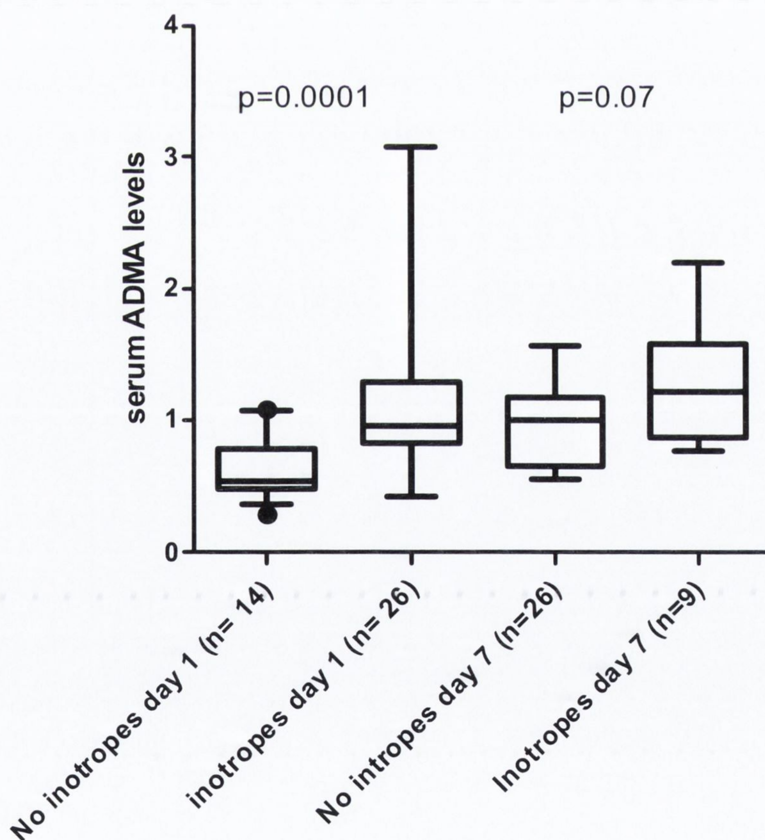
Plasma lactate levels directly correlated with ADMA levels on day 1 (spearman's rho = 0.53, n = 40, $p=0.0003$). Also, SOFA score and ADMA levels directly correlated on day 1 ($p<0.0001$, figure 7.2A).

In order to elucidate whether the relationship between ADMA levels and SOFA score was entirely attributable to cardiovascular failure a non-cardiac organ failure score was obtained by excluding the cardiovascular component from the total

SOFA score. There was a positive correlation between this score and ADMA levels on day 1 (Spearman's Rho =0.48, n=40, p =0.002).

ADMA levels on day 1 were not related to survival. In order to assess whether the highest producers of ADMA had a worse outcome, ADMA levels were divided into quartiles and patients in the highest quartile were compared to the remaining patients. These high producers of ADMA (highest quartile) were not more likely to have a higher mortality. However, SOFA scores on day 1 did distinguish between survivors and non-survivors on day 1 (p=0.02) (table 7.1).

Figure 7.1



ADMA levels and requirements for inotropes. Values, represented as box plots, are median, quartiles 25% to 75% (box), and centiles 10% to 90% (whiskers). p values are comparisons between the "inotropes" and the "no inotropes groups". ADMA measured as μmol/L.

Table 7.1: Demographics and ADMA levels by group

	Survivors	Non-survivors	p value
N	33 (70)	14 (30)	
Male sex	17 (52)	9 (64)	ns
SOFA score day 1	7 (4-10)	9 (8.75 – 12.5)	0.02
SOFA score day 7	4 (2.75 – 7.25)	10.5 (8.5 – 11.75)	0.006
SAPS2 score	39 (30.5 – 51.5)	47.5 (39.5 – 60.75)	ns
Lactate day 1	2.1 (1.5 – 5)	3.6 (2.25 – 5.75)	ns
Lactate day 7	1.5 (1- 1.9)	0 (1.1 – 4)	ns
Inotropes day 1	18 (55)	13 (93)	0.01
Inotropes day 7	5 (15)	5 (63)	0.005
ADMA day 1	0.88 (0.52 – 1.09)	0.91 (0.64 – 1.23)	ns
ADMA day 7	1.05 (0.66 – 1.21)	1.24 (0.77 – 1.53)	ns
pH day 1	7.31 (7.26-7.38)	7.33 (7.27-7.37)	ns
pH day 7	7.44 (7.40-7.45)	7.32 (7.24-7.40)	0.002
WCC day 1	18 (10.7-23)	9.1 (2.3-17.8)	ns
WCC day 7	11.6 (8.6-17.4)	13.9 (8.5-19.3)	ns
Base excess day 1	-2.9 (-6.45-1.75)	-4.1 (-9.3-1.75)	ns
Base excess day 7	2.9 (1.1-5.6)	0.1 (-3-1.33)	0.009

All values either as an absolute count with percentage in parenthesis or as median with interquartile range in parenthesis. ADMA measured in µmol/L. Lactate measured in mg/dL. SOFA, sequential organ failure assessment score; SAPS, simplified acute physiology score; ADMA, asymmetrical dimethyl arginine; WCC, white cell count; ns, non-significant.

Table 7.2: Requirement for inotropes on day 1

	Inotropes	No inotropes	p value
N	31 (66)	16 (34)	
Death	13 (42)	12 (6)	0.01
pH	7.31 (7.26 – 7.35)	7.32 (7.29 – 7.40)	ns
Lactate	3.5 (1.9 – 6.02)	1.8 (1.05 – 3.75)	0.02
Base excess	-3.5 (-9-3.4)	-2 (-4.95-0.55)	ns
SOFA	9 (8-12)	4 (3.25-4.75)	<0.0001
SAPS2	47 (38-63)	33.5 (21-41.75)	0.003
ADMA	0.96 (0.82-1.29)	0.54 (0.48-0.78)	0.001
WCC	14 (8-22)	17 (8-24)	ns

All values either as an absolute count with percentage in parenthesis or as median with interquartile range in parenthesis. ADMA measured in μmol/L. Lactate measured in mg/dL. SOFA, sequential organ failure assessment score; SAPS, simplified acute physiology score; ADMA, asymmetrical dimethyl arginine; WCC, white cell count; ns, non-significant.

On day seven 10 patients (24%) required an inotrope to maintain a normal blood pressure. Whilst there was a trend towards increasing ADMA levels in those patients requiring inotropes to maintain blood pressure this did not reach significance ($p = 0.07$, figure 7.1 and table 7.3).

Plasma lactate levels directly correlated with ADMA levels on day 7 (spearman's rho = 0.42, n = 31, p=0.01). Also, SOFA score and ADMA levels directly correlated on day 7 (p=0.002, figure 7.2B). The non-cardiac organ failure

score was calculated as above from the day 7 SOFA score. This score positively correlated with day 7 ADMA levels (spearmans rho =0.47, n=35, p=0.005).

ADMA levels on day 7 were not related to survival, nor were the highest producers of ADMA (highest quartile) more likely to have a higher mortality. However, increased SOFA scores, acidosis and requirement for inotropes on day 7 were associated with increased risk of death (table 7.1).

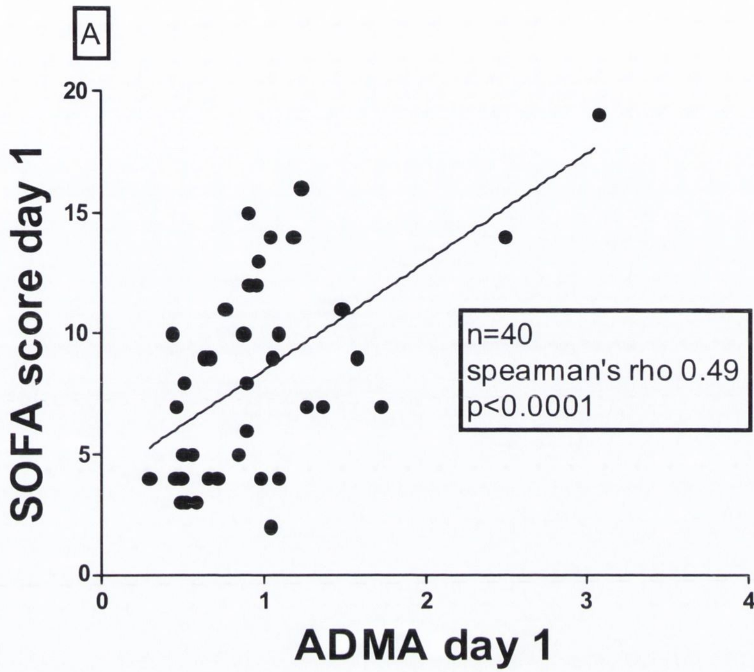
On the first day of critical illness the ICU group had greater ADMA levels than did the control group (p=0.005). ADMA levels subsequently rose over the first week in the ICU group (p=0.001, figure 7.3).

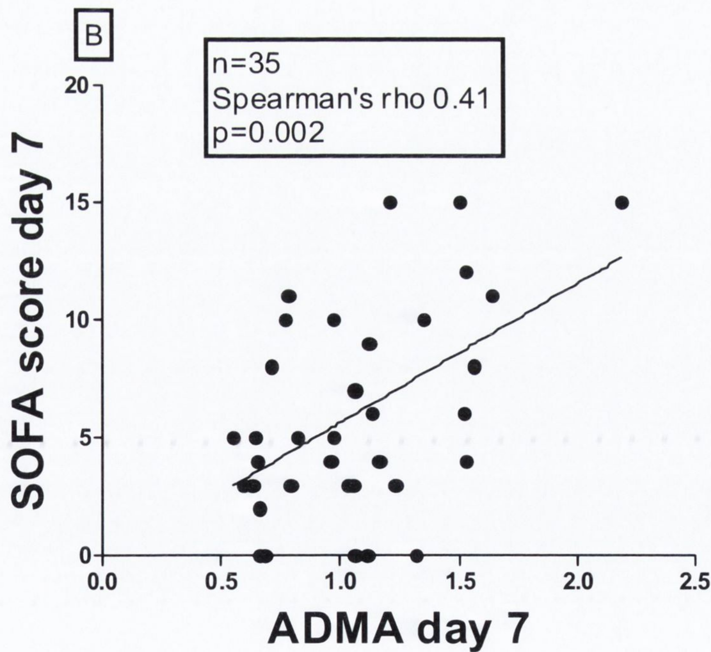
Table 7.3: Requirement for inotropes on day 7

	Inotropes	**No inotropes**	**p value**
N	10 (24)	31 (76)	
Death	5 (50)	3 (10)	0.005
PH	7.36 (7.23-7.41)	7.44 (7.40-7.45)	0.0006
Lactate	2.35 (1.48-3.73)	1.2 (1-1.8)	0.006
Base excess	0.05 (4.7-3.8)	2.9 (1.1-4.8)	0.04
SOFA	11 (10-15)	4 (2.3-5.8)	<0.0001
ADMA	1.21 (0.88-1.57)	1 (0.66-1.18)	ns
WCC	18.2 (13.3-22.2)	10 (8.2-13.8)	0.01

All values either as an absolute count with percentage in parenthesis or as median with interquartile range in parenthesis. ADMA measured in μmol/L. Lactate measured in mg/dL. SOFA, sequential organ failure assessment score; ADMA, asymmetrical dimethyl arginine; WCC, white cell count; ns, non-significant.

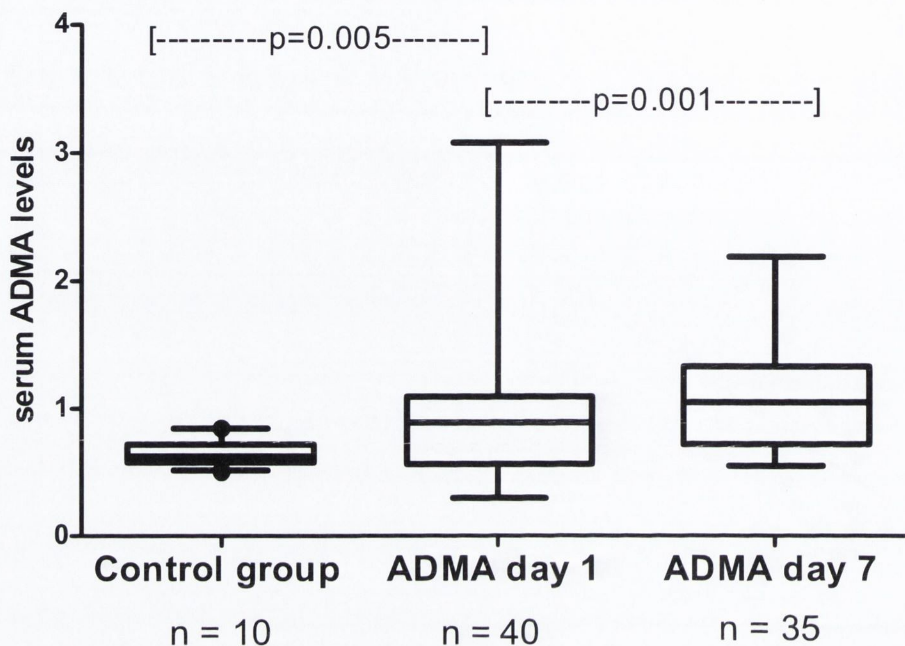
Figure 7.2





Correlation between ADMA and SOFA score. There was a direct correlation between ADMA and SOFA score on both day 1 (A) and on day 7 (B) of ICU stay. ADMA measured as μmol/L.

Figure 7.3



ADMA levels in the controls and on day 1 and day 7 in the ICU group. Values, represented as box plots, are median, quartiles 25% to 75% (box), and centiles 10% to 90% (whiskers). p values are comparisons between the controls and ADMA on day 1 and between ADMA on day 1 and on day 7. ADMA measured as μmol/L.

Various inflammatory markers correlated with ADMA levels on univariate analysis on both day 1 and day 7 (table 7.4). Whilst pH, base excess and lactate levels correlated with ADMA on both day 1 and day 7, the white cell count (WCC) did not correlate with ADMA at either time point (table 7.4).

Table 7.4: Correlation matrix of ADMA and inflammatory markers

	ADMA day 1 data	**ADMA day 7 data**
pH	0.31 (0.0002)	0.32 (0.001)
Base excess	0.13 (0.02)	0.24 (0.005)
Lactate	0.29 (0.0003)	0.20 (0.01)
WCC	ns	ns

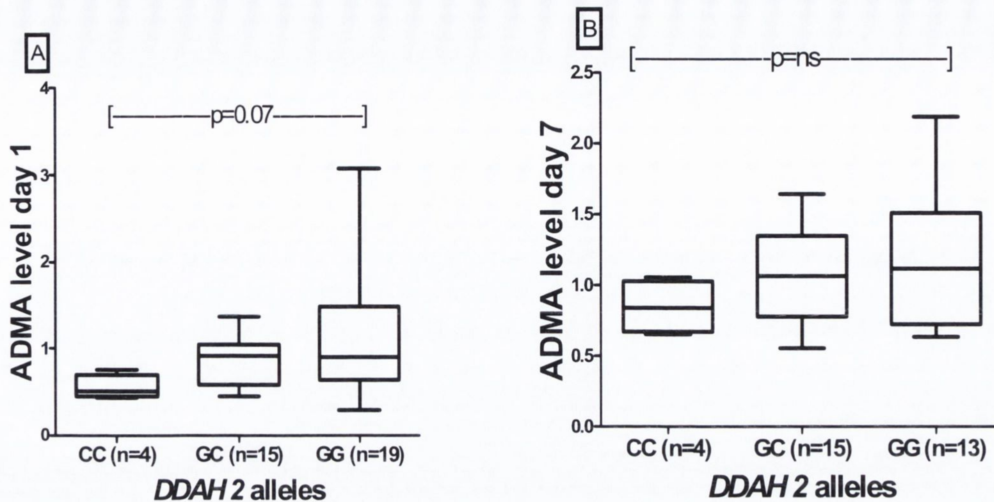
Values are r^2 with p values in parenthesis. WCC, white cell count; ns, non significant. Lactate as mg/dL.

7.2.2 Influence of a polymorphism in *DDAH II* on ADMA levels

DDAH is an enzyme whose primary function is to metabolise ADMA. This enzyme exists as two isoforms, with DDAH II found primarily in tissues that express eNOS.

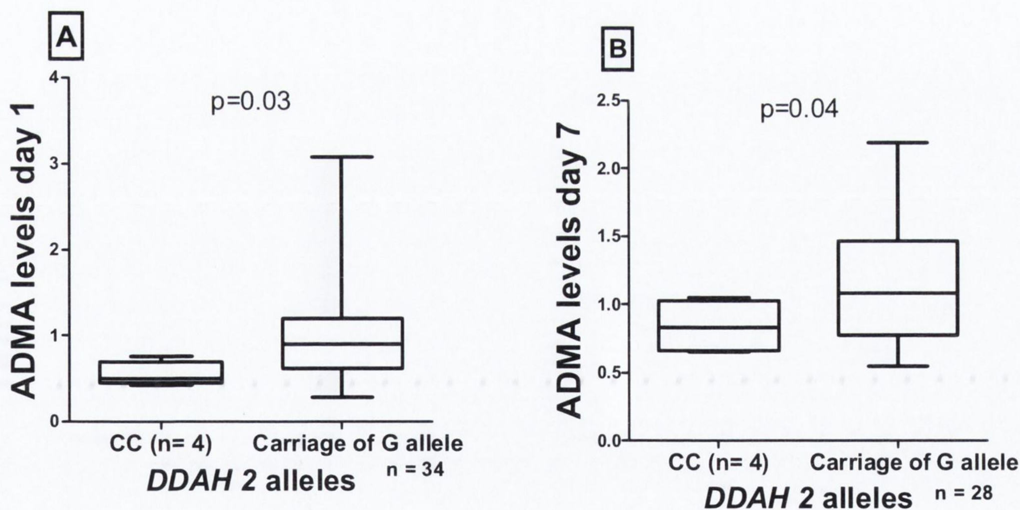
The distribution of *DDAH II* –449 alleles conformed to a Hardy Weinberg equilibrium. We failed to observe any association between clinical outcome measures and carriage of specific *DDAH II* alleles. Twenty-four (45%) patients were GG homozygotes, 5 (11%) were CC homozygotes and 19 (43%) were hetereozygotes at position –449 in the *DDAH II* promoter. There was a trend towards increasing amounts of ADMA between different *DDAH II* genotypes. ADMA was most abundant in the GG homozygotes, least abundant in the CC homozygotes and detectable at intermediate levels in the heterozygotes. This trend was present at both time points although it failed to reach significance on either day 1 ($p = 0.069$) or on day 7 ($p = 0.32$) (figure 7.4). However, carriage of the G allele at position –449 was associated with increased ADMA production on both day 1 ($p = 0.03$) and 7 ($p = 0.04$) (Figure 7.5).

Figure 7.4



ADMA levels and DDAH II –449 genotypes. A ADMA levels on day 1 of ICU stay and DDAH II genotypes. B ADMA levels on day 7 of ICU stay and DDAH II –449 genotypes. Values, represented as box plots, are median, quartiles 25% to 75% (box), and centiles 10% to 90% (whiskers). p values refer to a 3 group comparison. ADMA measured as μmol/L.

Figure 7.5



ADMA levels and carriage of specific alleles at DDAH II –449. ADMA levels on day 1 (A) and day 7 (B) of ICU admission. Comparison is between CC homozygotes and patients carrying a G allele (either GG or GC) at position -449. Values, represented as box plots, are median, quartiles 25% to 75% (box), and centiles 10% to 90% (whiskers). ADMA measured as μmol/L.

7.3 IL-6 protein levels

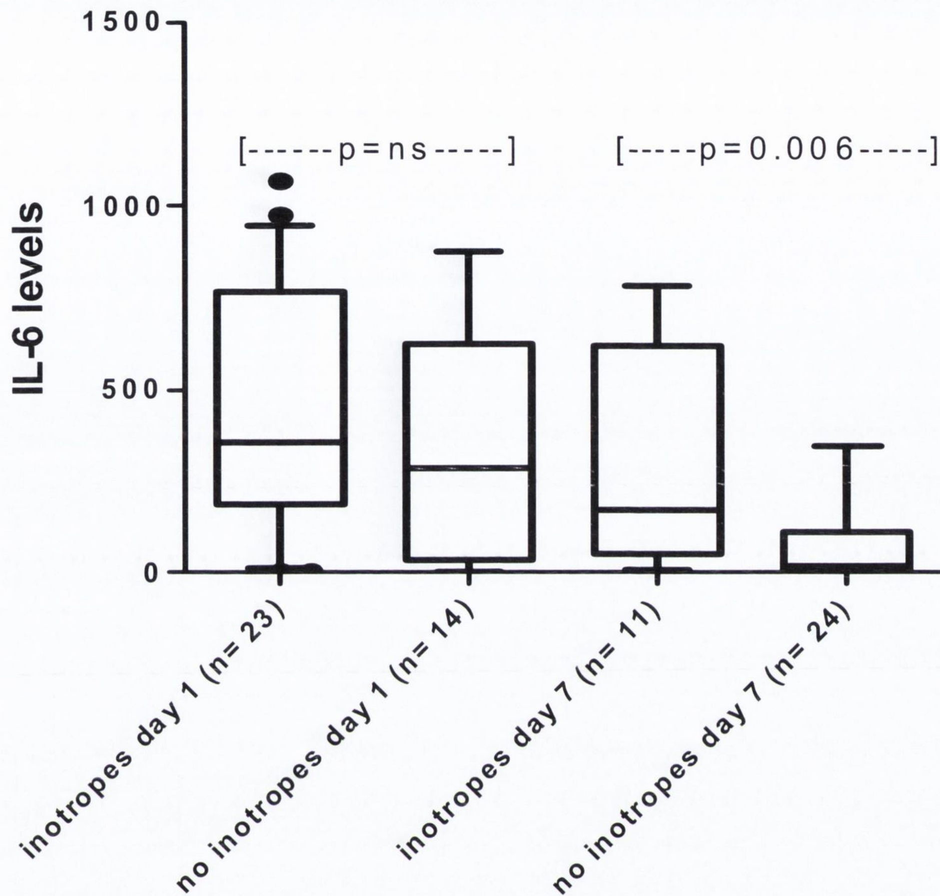
7.3.1 Description of IL-6 protein levels in the ICU and control groups

Serum was available for analysis of IL-6 by ELISA in 37 patients on day 1 and in 36 patients on day 7. In 28 patients IL-6 was available for analysis at both time points. On day 1 median IL-6 levels were 305 (IQR 131 – 732, all IL-6 measurements as pg/ml). On day 1, 4 patients (11%) had undetectable IL-6 protein. Median IL-6 levels on day 7 were 64 (IQR 4 – 174). On day 7, 7 patients (19%) had undetectable IL-6 protein. The difference in IL-6 levels from day 1 to day 7 was non significant (p=0.3).

On day 1, IL-6 protein levels did not distinguish between shocked and non-shocked patients (figure 7.6). Whilst, IL-6 levels on day 1 were not directly associated with the pH, there was a direct relationship with base excess (n=37, spearman's rho 0.52, p=0.001) and with lactate levels (n=37, spearman's rho 0.33, p=0.04). There was also a direct relationship between IL-6 levels and the SAPS2 score (n=37, spearman's rho 0.38, p=0.02) and the SOFA score (n=37, spearman's rho 0.37, p=0.03).

On day 7, median IL-6 protein levels were higher in the shocked patients compared to the non-shocked patients (p=0.006, figure 7.6). There was no relationship detected between IL-6 protein and acidosis, lactate levels or organ failure scores on day 7.

Figure 7.6



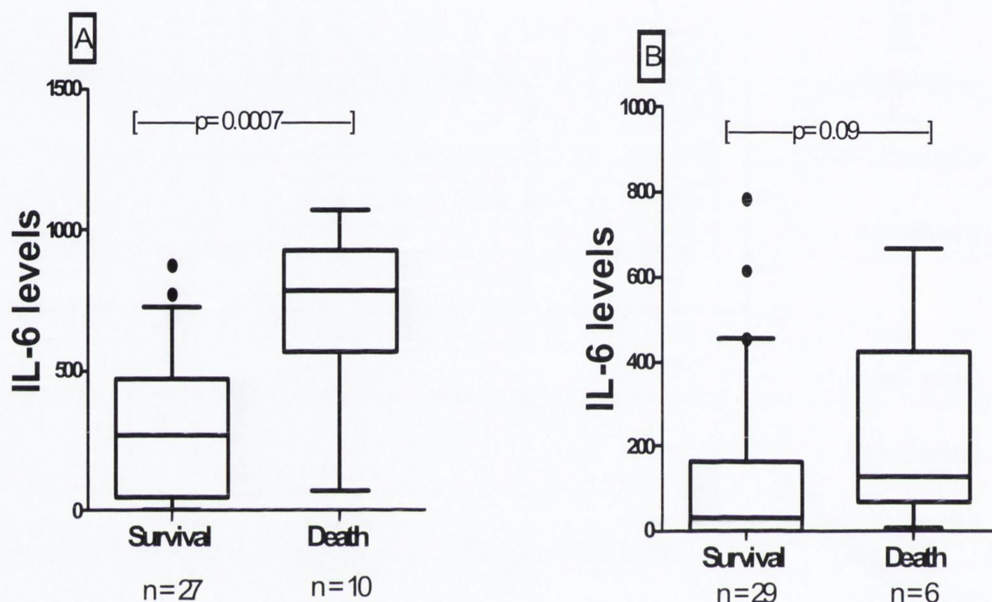
IL-6 protein levels and requirement for inotropes. p values are for comparison of each of the pairs "inotropes" and "no inotropes". Values, represented as box plots, are median, quartiles 25% to 75% (box), and centiles 10% to 90% (whiskers). IL-6 measured as pg/ml.

On day 1 higher IL-6 levels were associated with an increased risk of subsequent death in the ICU (p=0.0007, figure 7.7A). While the trend remained on day 7 the result was not statistically significant (p=0.09, figure 7.7B).

On the first day of critical illness there was a negative correlation between IL-6 protein levels and IFNγ mRNA levels (Spearman's Rho = -0.52, p=0.0009). On day 7 there was a negative correlation between IL-6 protein levels and TNFα mRNA levels (Spearmans Rho = -0.43, p=0.006). Also, on day 7, IL-6 protein levels were greater in ICU patients in the cluster with lesser TNFα and IFNγ mRNA production

(table 4.4). Patients in cluster 1 had median IL-6 levels of 10.7 (0-103) whilst in cluster 2 median IL-6 levels were 142 (29 – 456) (p=0.007).

Figure 7.7



IL-6 levels and survival in the ICU group. A IL-6 levels measured on day 1 were higher in patients who subsequently died in the ICU. B There was a non significant trend for IL-6 levels measured on day 7 of ICU stay to be higher in patients who subsequently died. Values, represented as box plots, are median, quartiles 25% to 75% (box), and centiles 10% to 90% (whiskers). IL-6 measured as pg/ml.

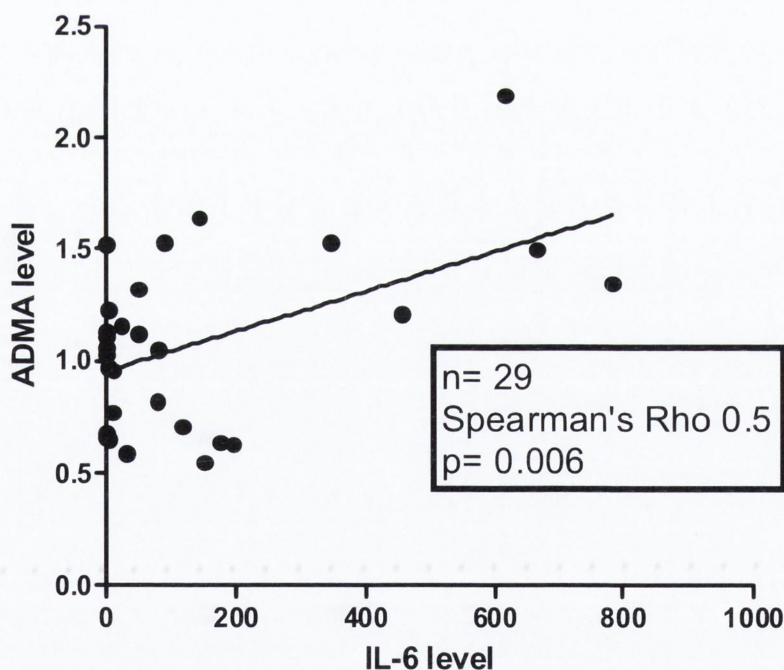
From the control group 7 subjects had serum analysed for the presence of IL-6. Only 2 subjects had detectable levels of IL-6 protein with the average amount being 139pg/ml.

7.3.2 Correlation between IL-6 and ADMA and between severity of organ failure and IL-6 and ADMA levels

On a univariate analysis IL-6 and ADMA correlated on day 7 ($p=0.006$, figure 7.8) but not on day 1.

Multivariate analysis of the relationship between the SOFA scores and the biologic markers, ADMA and IL-6, revealed that on day 1 both ADMA ($p=0.002$) and IL-6 ($p=0.009$) were independently related to SOFA scores, while on day 7 only ADMA ($p=0.002$) was independently related to the SOFA score (Table 7.5).

Figure 7.8



Correlation between IL-6 and ADMA on day 7 of ICU stay. IL-6 as pg/ml and ADMA as μmol/L.

Table 7.5: Multivariate linear regression between SOFA scores and ADMA and IL-6

Day 1	**F ratio**	**p value**
ADMA	11	0.002
IL-6	7.8	0.009
N=36, $r^2 = 0.35$		
Day 7		
ADMA	12.02	0.002
IL-6	0.88	Ns
N = 30, $r^2 = 0.32$		

SOFA, sequential organ failure assessment score; ADMA, asymmetrical dimethyl arginine; IL-6, interleukin 6; ns, non significant

7.3.3 Influence of an IL-6 promoter polymorphism on IL-6 levels

The distribution of *IL-6* –174 alleles conformed to Hardy Weinberg equilibrium. We failed to observe any association between clinical outcome measures and carriage of specific *IL-6* alleles. 10 (15%) patients were CC homozygotes, 16 (27%) were GG homozygotes and 34 (54%) were heterozygotes at position -174 in the *IL-6* promoter (table 7.6). IL-6 genotype was not associated with differing amounts of IL-6 protein (table 7.6). Additionally, neither the CC nor GG homozygotes were associated with altered IL-6 protein when compared to the other genotypes combined.

Table 7.6: IL-6 protein levels Vs IL-6 -174 genotype

	CC	GC	GG	p value
N	10 (15)	34 (54)	33 (27)	
IL-6 day 1	160(0 - 740)	323(217 - 797)	293(136 - 773)	ns
IL-6 day 7	456(0-784)	39(4 - 124)	79(13 - 157)	ns

Values are either absolute counts with percentages in parenthesis or median with interquartile range in parenthesis. IL-6 as pg/ml. Groups compared by Kruskal-Wallis test.

7.4 TNFα, IL-10 and IFNγ protein levels

On day 1, in the ICU group TNFα protein was detectable in 14 of the 35 patients (40%) that had serum analysed. The median value was 0 and the mean 67 (all values in pg/ml). On day 7, TNFα protein was detectable in 9 of the 33 patients (28%) analysed. The median value was 0 and the mean 59.

On day 1, IL-10 protein was detectable in 14 of 36 patients (38%). The median was 0 and the mean 63. On day 7, Il-10 protein was detectable in 13 out of 33 patients (39%). The median was 0 and the mean 10.

On day 1, IFNγ protein was detectable in 19 out of 36 patients analysed (53%). The median value was 0.69 and the mean was 4.58. On day 7, IFNγ protein was detectable in 18 out of 33 patients (54%). The median value was 0 and the mean value was 4.45.

Protein levels were checked for any correlation with the corresponding mRNA levels. No correlation was detected. No relationship was detected between TNFα, IL-10 or IFNγ and any of the clinical outcome variables.

7.5 Discussion

As ADMA is a potent NOS inhibitor and given the ubiquitous involvement of NO in vascular regulation and leukocyte function, the consequences of excess ADMA in inflammatory and septic states are likely to be manifold. There are, however, limited data on the role of ADMA and DDAH II in systemic inflammation, with two studies of critically ill patients observing a relationship between the highest producers of ADMA and fatal outcome (Nijveldt et al., 2003a; Siroen et al., 2005). We followed the method of analysis used in these studies and divided the ADMA levels into quartiles. However, we failed to find an association between the highest producers (highest quartile) and outcome as had been previously described (Nijveldt et al., 2003a). Whilst our study may not have been adequately powered to detect outcome variations, we have demonstrated both an increase in ADMA levels in critically ill patients in comparison to healthy controls, and described an association between increasing ADMA levels, the occurrence of septic shock and greater severity of organ failure.

Potentially, in inflammatory states, raised ADMA levels may lead to pathogenic changes in the micro-vasculature by inhibiting constitutively expressed NOS (Nijveldt et al., 2003b). The consequent loss of basal NO production may lead to impaired blood flow with platelet aggregation causing endothelial damage, interstitial oedema and resultant organ failure (Vallance, 2001).

However, ADMA mediated iNOS inhibition in septic patients may interfere with macrophage bactericidal properties as NO is an essential component in the phagocytic response to bacterial infection. IFNγ, released in response to an infective insult, acts on macrophages to increase expression of iNOS (Boehm et al., 1997). This activates the cells to a heightened microbicidal state, mediated by NO and adducts of

the nitrogenous products of nitric oxide synthases. As a consequence mice with a non-functional iNOS gene are susceptible to infection (Wei et al., 1995). Furthermore, in clinical trials of NOS inhibition in septic patients, although NOS inhibition ameliorates pathogenic vasodilation and lessens vasopressor requirement, the overall effect is to compromise survival (Lopez et al., 2004). This suggests, in the context of severe sepsis, that NO linked immune mechanisms are of greater importance that NO meditated vascular regulation.

We observed that elevated ADMA levels correlate with vasopressor support in early septic shock. Whilst this may seem counterintuitive, as previous evidence implicated NO in the pathogenesis of the hypotension observed in septic shock (Brady and Poole-Wilson, 1993), it is plausible that inappropriately increased ADMA levels may impair macrophage function via NOS inhibition. The associated inflammatory response to an unresolved infection may be partly responsible for the observed hypotension and organ failure operating via an alternative mechanism. This persistent inflammatory response is reflected in the linkages between IL-6, ADMA and the severity of organ failure. The association with IL-6 is noteworthy as this is a well-recognized marker of generalized inflammation, consistently elevated in septic patients (Watanabe et al., 2005).

Approximately 90% of ADMA is metabolised by the enzyme DDAH (Tran et al., 2003). It is possible that variation in ADMA levels in septic patients is reactive and represents an epi-phenomenon. However, we observed that carriage of a G allele at position –449 in the promoter region of the *DDAH II* gene is associated with increased ADMA levels, which suggests that the *DDAH II* gene with a G allele at position –449 is less active than that with a C allele. The more active isoform results in lower ADMA levels, less iNOS inhibition and consequently appropriate

bactericidal phagocytic response. It is noteworthy that *DDAH II* maps to 6p21.3, in the MHC, a region of the genome that is particularly rich in genes involved in immune and inflammatory responses. It has been hypothesised that this location and wide expression in immune cells make *DDAH II* a candidate as a disease susceptibility gene in sepsis (Tran et al., 2003).

We have previously described an association between the presence of the G allele at position –449 in the *DDAH II* gene and the requirement for vasopressors after cardiopulmonary bypass (CPB) during cardiac surgery (Ryan et al., 2006). Whilst this is the opposite of what we observed in septic patients, it is noteworthy that the two insults are also quite different. The CPB circuit invokes a sterile inflammatory response whereas the septic ICU patients received an infective inflammatory insult. Consequently, ADMA's role in manipulating NO levels may be context sensitive. NO may have pivotal beneficial bactericidal properties necessary for the resolution of a septic insult whilst contributing to an undesirable vasodilatory state in the setting of a sterile inflammatory insult.

This potential genetic component to the fluctuations observed in ADMA levels secondary to a septic insult may help to explain some of the residual variability observed in a previous study attempting to link exogenous insulin administration to ADMA levels (Siroen et al., 2005). Thus, inter-individual variability in ADMA production is likely to be multifactorial, with contributions from genetic and environmental factors.

We have found that greater IL-6 levels were associated with lesser TNFα and IFNγ mRNA levels in a patient group with poor outcome. IL-6 is a Th2 cytokine, it activates the acute phase response and controls switching of immunoglobulin subclasses (Kishimoto, 2005). Despite these pro-inflammatory actions, IL-6 does not

appear to possess significant bactericidal activity, as witnessed by IL-6 knockout mice not experiencing excess mortality in animal models of peritonitis (Leon et al., 1998; Remick et al., 2005). Yet IL-6 production is undoubtedly of importance in patients with sepsis, as high IL-6 levels are predictive of excess mortality, with certain IL-6 haplotypes linked with greater IL-6 production and greater severity of organ failure in patients with severe sepsis (Sutherland et al., 2005).

However, IL-6 also acts to modulate the balance between the Th1 and Th2 response to infection, promoting the Th2 response via both IL-4 dependant and independent mechanisms, and by inhibiting the Th1 response (Diehl and Rincon, 2002). As IFNγ is the prototypic Th1 cytokine, with a pivotal role in generating cell mediated bactericidal activity, IL-6 may actually impair phagocytic bactericidal activity by inhibiting IFNγ production. This particular phenomenon is specifically evident in the context of mycobacterial disease where IL-6 has been demonstrated to inhibit IFNγ production and associated bactericidal activity (Diehl and Rincon, 2002; Nagabhushanam et al., 2003). Moreover, this may represent the basis for the linkage of greater IL-6 protein levels with lesser TNFα and IFNγ mRNA observed in our patients with persistent shock and greater mortality. Thus, IL-6 may generate excess inflammation while simultaneously impairing bactericidal activity and it is plausible that this is why excess IL-6 production is linked to greater severity of illness and an increased mortality.

The functionality of the –174 SNP in IL-6 has been reported previously. The C allele has been associated with reduced IL-6 production both in reporter gene assays and in plasma from healthy controls (Lin and Albertson, 2004). We failed to see any effect of this polymorphism on IL-6 levels in our cohort but the study may have been underpowered to detect such an effect. However, a number of other clinical studies

have also failed to find any association of the IL-6 promoter SNP to IL-6 levels *in vivo* (Lin and Albertson, 2004).

Quantification of cytokine production using ELISA is a well-established technique. However, much of the early work in sepsis relied on rodent and primate models of overwhelming endotoxemia or bacteraemia. There are however concerns about the sensitivity of the method in human sepsis *in vivo*. Previous studies in patients with sepsis report detectable TNFα in fewer than 10% of samples in some cases (Oberholzer et al., 2000). We can confirm these previous observations that ELISA is an insensitive technique for quantifying TNFα, IL-10 or IFNγ production *in vivo*. It is interesting that even in those patients with detectable protein there was no correlation with the results obtained using QRT-PCR.

7.6 Conclusion

We have confirmed the association between ADMA levels and the extent of multiple organ failure in sepsis. We have also demonstrated that ADMA levels are upregulated in response to an infective insult and are also associated with hypotension in this setting. We hypothesise that this may be due to ineffectual bactericidal activity of macrophages and persistent inflammation. Finally, we suggest that ADMA levels may be regulated via a genetic component. We propose that a polymorphism at position –449 in the *DDAH II* may be functional and has the potential to be used as a marker for the susceptibility to and severity of an inflammatory response secondary to an infective insult. A larger study will be required to confirm these findings.

Chapter 8 - Results (6): Characterisation of genetic variants influencing gene expression in patients with severe sepsis

8.1 TNFα genetic variants

8.1.1 TNFα promoter SNPS

The polymorphic sites in the TNFα promoter region assessed were a G/A SNP at position –238, a G/A SNP at position –308, a C/T SNP at position –857 and a C/A SNP at position –863. The distribution of TNFα alleles conformed to a Hardy Weinberg equilibrium and allele frequencies are given in table 8.1.

There was no association between the SNPs at positions –238 and –857 and TNFα mRNA levels at either time point. Additionally, there was no association between either of these two SNPs and any of the clinical correlates assessed.

Patients carrying an A allele at position –863 (n=17) produced more TNFα mRNA on day 1 than did C homozygotes (p=0.037 figure 8.1A). There was a non-significant trend for patients homozygous for the G allele at position –308 (n=35) to produce more TNFα mRNA on day 1 than those carrying an A allele (p=0.059, figure 8.1B).

No patient carrying an A allele at position –863 also carried an A allele at position –308 (haplotypes are shown in figure 8.2). The relationship between SNPs and TNFα mRNA levels was analysed by comparing all patients with an A allele at position –308, all patients with an A allele at position –863 and all other variants as a 3 group comparison. On day 1, the relationship between the SNPs and TNFα mRNA was of borderline significance with the A allele at –863 being the high producer, the A allele at -308 the low producer and the remaining patients being intermediate producers (p=0.06, figure 8.2A).

However, upon direct comparison between patients with an A allele at position –308 and patients with an A allele at position –863, the presence of an A allele at -863

was associated with greater levels of TNFα mRNA when compared with patients carrying the A allele at -308 on day 1 (p=0.02, figure 8.2B).

These associations did not persist on day 7.

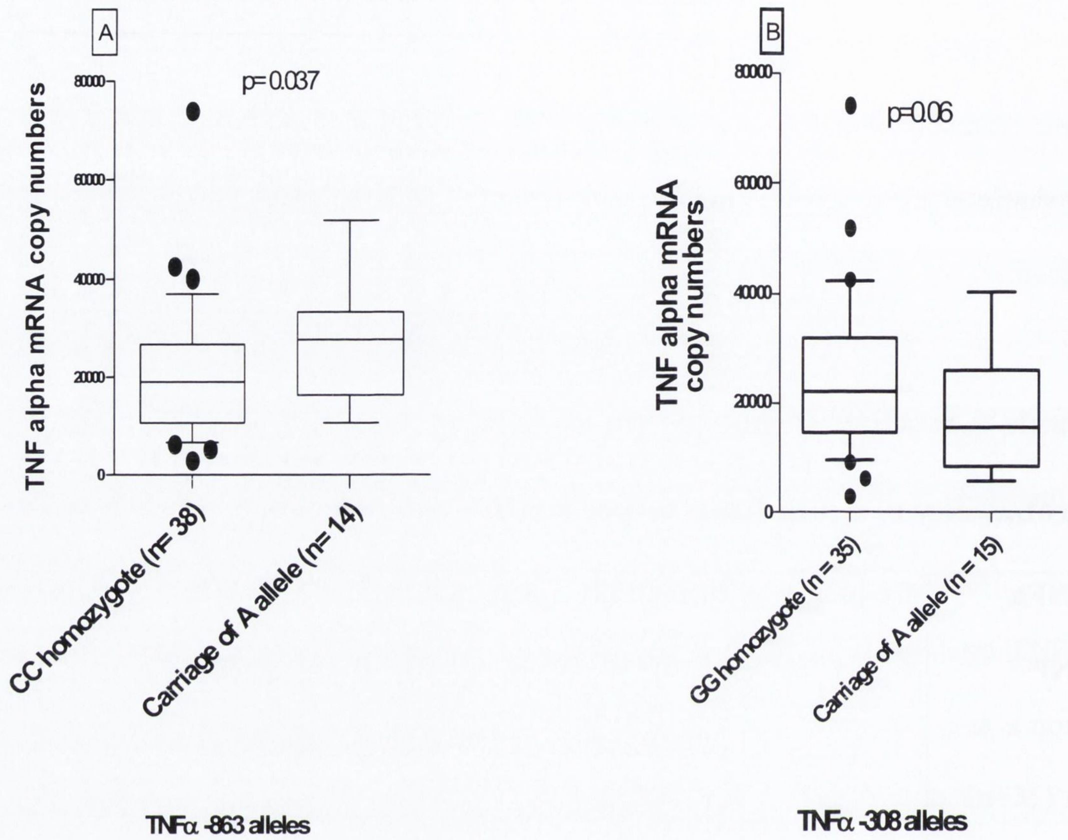
Patients homozygous for the A allele at position –308 were more likely to succumb to severe sepsis than those carrying the G allele (p=0.01, table 8.1).

Table 8.1: Allele frequency for specific promoter SNPs in relation to probability of death from severe sepsis.

TNFα SNP	**frequency**	**Probability of death if homozygous for minor allele**	**Probability of death if heterozygous**	**Probability of death if minor allele absent**	**p value**
-308 G/A	0.64/0.36	1.0	0.1	0.31	0.01
-238 G/A	0.87/0.13	N/A	0.25	0.38	ns
-857 C/T	0.87/0.13	0	0	0.3	ns
-863 C/A	0.72/0.28	0.5	0.33	0.23	ns

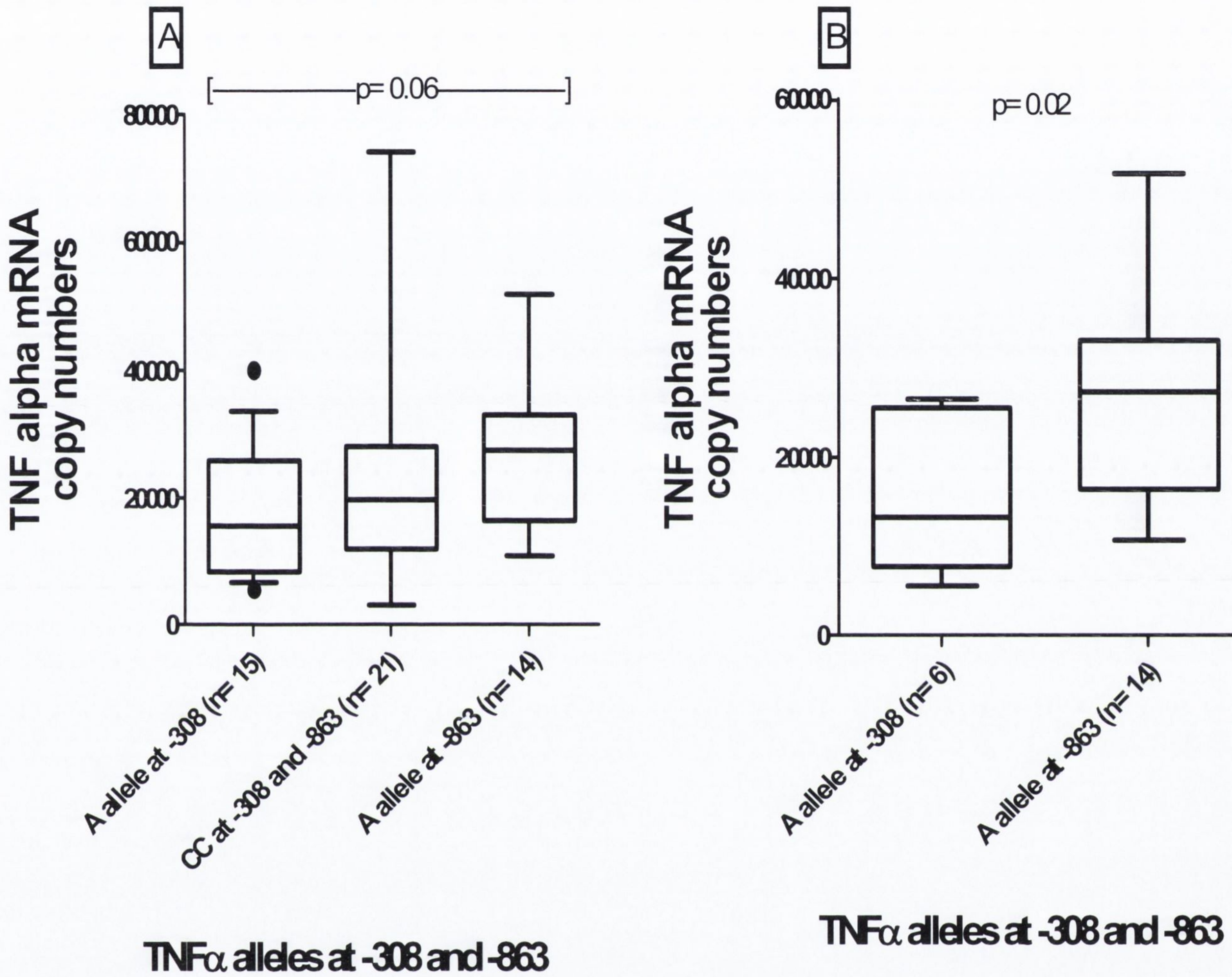
The minor allele refers to the less frequently occurring allele. N/A, non applicable as no homozygotes present. Ns, non significant. Comparison by Kruskal-Wallis test.

Figure 8.1



TNFα mRNA levels on day 1 in the ICU group and specific alleles in the TNFα promoter. Values, represented as box plots, are median, quartiles 25% to 75% (box), and centiles 10% to 90% (whiskers). The comparisons are C homozygotes versus all other genotypes at –863 (A) and between G homozygotes and all other genotypes at –308 (B).

Figure 8.2



Different TNFα promoter SNPs compared in terms of TNFα mRNA production on day 1 in the ICU group. Values, represented as box plots, are median, quartiles 25% to 75% (box), and centiles 10% to 90% (whiskers). Figure A represents a three group comparion and figure B a 2 group comparison.

8.1.2 TNFα promoter haplotypes

Statistical confidence for assigned haplotypes were >95% for all individuals but one, which was then removed from the subsequent analysis. Carrier status for haplotype 1 (table 8.2) was associated with greater TNFα mRNA levels on day 1 when compared to all other haplotypes (p=0.04). Distribution of mRNA values and the p value obtained was identical to that observed using the –863 SNP alone. Similarly, an analysis of carrier status for haplotype 4 and TNFα mRNA levels revealed identical results as was seen for the –308 SNP, with a borderline association between carriage of haplotype 4 and lesser TNFα mRNA levels in comparison to all other haplotypes (p=0.06). No carriers of both haplotypes 1 and 4 were identified in this study. Direct comparison of TNFα production between haplotype 1 and haplotype 4 again revealed identical results to that obtained when analysing the SNPs alone at positions –863 and –308. All loci conformed to Hardy-Weinberg Equilibrium and the observed allele frequencies are consistent with those reported on the dbSNP and Hapmap databases. In addition, frequencies of TNF-863/-308 haplotypes are similar to those reported on Hapmap for the CEPH European population. Because this latter population consists of 30 trios of both parents and one offspring, the haplotypes can be observed directly in most cases. While our haplotype frequencies were inferred computationally using PHASE, the similarity with HapMap data suggests that they are reliable.

On analysis of the data using haploscore (Schaid et al., 2002) we failed to find an association between TNFα production on day 1 and haplotype (global score statistic 5.3, degrees of freedom (df) 4, p=0.26). Similarly, on day 7 there was no association between haplotype and TNFα mRNA using haploscore (global score statistic 4.42, df 4, p=0.35).

Table 8.2: Frequencies of the observed TNFα promoter haplotypes and association with TNFα mRNA production on day 1.

Haplotype	-238	-308	-857	-863	Frequency	P value
1	G	G	C	A	0.153	0.04*
2	G	G	C	C	0.516	
3	G	G	T	C	0.073	
4	G	A	C	C	0.194	0.06¶
5	A	G	C	C	0.064	

A, adenine; C, cytosine; G, guanine; T, thymidine. Frequencies of all haplotypes = 1.0

*As haplotype 1 alone possessed an A allele at position –863 this haplotype was assessed for association with TNFα mRNA production on day 1 and was found to be associated with greater TNFα mRNA levels when compared to all other haplotypes (p=0.0374, comparison by Wilcoxon rank sum test).

¶ As haplotype 4 alone possessed an A allele at position -308 this haplotype was assessed for association with TNFα mRNA production on day 1 and was found to be associated with non-significantly lesser TNFα mRNA levels when compared to all other haplotypes (p=0.059).

8.2 IL-10 genetic variants

The polymorphic sites in the IL-10 promoter region assessed were a C/A SNP at position –592, a C/T SNP at position –819 and a G/A SNP at position –1082. The distribution of IL-10 alleles conformed to a Hardy Weinberg equilibrium. At the –592 site 2 patients (3%) were A homozygotes, 33 patients (57%) were C homozygotes and 23 patients (40%) were heterozygotes. At the –819 site 35 patients (58%) were C homozygotes, 2 patients (3%) were T homozygotes and 23 patients (38%) were heterozygotes. At the –1082 site 11 patients (18%) were A homozygotes, 16 patients (27%) were G homozygotes and 33 patients (55%) were heterozygotes.

Using standard statistical association tests (Kruskal-Wallis test and Wilcoxin test), no association was detected between any of the IL-10 genotypes and IL-10 mRNA levels on either day 1 or day 7 in the ICU group. No association was detected between genotypes and any clinical correlates.

Similarly, using haploscore no association was detected between IL-10 haplotypes and IL-10 mRNA levels on either day 1 (global score statistic 0.06, df 2, p=0.97) or on day 7 (global score statistic 0.52, df 2, p=0.77).

8.3 PAI-1 polymorphisms in the ICU group

Patients were analysed for the presence of a 4G/5G polymorphism in the promoter region of the PAI-1 gene. The distribution of PAI-1 alleles conformed to a Hardy Weinberg equilibrium. 22 patients (35%) were 4G homozygotes, 18 patients (29%) were 5G homozygotes and 22 patients (35%) were heterozygotes.

There was no association between genotype and any of the clinical correlates. Similarly, when patients homozygous for the 4G allele were compared against all other genotypes no associations were detected.

8.4 Discussion

The majority of genetic studies in sepsis have focused on the influence of individual SNPs, commonly TNFα -308, on disease prevalence and gene function and have provided inconsistent results (Clark and Baudouin, 2006). However, recent genetic advances suggests that individual SNPs may have poor predictive power as the unique interactions of multiple SNPs within a haplotype may affect phenotype to a greater extent and therefore provide a more powerful tool to assess associations (Judson et al., 2000).

Our analysis shows that individual SNPs are better able to discriminate between low and high TNFα producers than when they are analysed together as a haplotype. This is most simply interpreted as indicating that the individual SNPs are the biggest determinants of TNF transcriptional activity. It is noteworthy that the high producing genotype containing an A allele at position –863 never exists in our cohort with an A allele at –308 (i.e. is in complete linkage disequilibrium with the -308G allele) while –308A is solely found with C homozygotes at position –863. Given that linkage disequilibrium between the sites is at a maximum (since only three of the four possible haplotypes are observed in this dataset) haplotype analysis will not add to the power of the individual SNP associations.

It may well be that the discordance between these data and previous publications (Clark and Baudouin, 2006), resulting in the labelling of the TNF2 allele as an overproducer of TNFα, are secondary to the method of quantification of *in vivo* gene expression. We propose that QRT-PCR provides more accurate and sensitive data when compared with ELISA based assays, performed in conjunction with *in vitro* LPS mediated amplification of cytokine gene expression. Indeed the extrapolation

from *in vitro* functional genetic tests after LPS stimulation may be of questionable value.

We did not observe an association between SNPs, haplotype structure and mRNA production in late sepsis. Following a septic insult it is likely that numerous factors influence TNFα gene expression. Unsurprisingly, genetic variation is of greater importance to TNFα gene expression at onset of a septic illness but may become overshadowed by other factors and interventions later in the course of the disease.

Whilst we did observe an association between outcome and carriage of the TNF2 allele, caution must be advised in the interpretation of this result in view of the low number of A homozygotes in our septic cohort. Although not consistent, other studies have described an association between the TNF2 allele and poor outcome in sepsis (Clark and Baudouin, 2006; Mira et al., 1999). It has been hypothesised that this genotype may be causative in increasing TNFα production, contributing to a pathogenically exuberant inflammatory response resulting in higher mortality in sepsis. This assumption, that TNFα plays an exclusively detrimental role in sepsis, has been based primarily on animal models (Tracey et al., 1986). The adverse outcome observed in clinical trials of anti-TNFα compounds in human sepsis serve to undermine this hypothesis and suggest a protective role for TNFα (Fisher et al., 1996). Crucially however, it is worth considering, from the data presented here, that the TNF2 allele may be linked with deficient TNFα production in sepsis, and it is this deficiency that may be pathogenic. We have already demonstrated in chapter 3 that deficiency of TNFα mRNA is associated with a poor outcome in sepsis.

We failed to observe any association between IL-10 genotypes or haplotypes and clinical correlates in our septic population. We have previously described an

association between IL-10 haplotypes and an inflammatory reaction post cardiopulmonary bypass in an Irish population using the same combination of promoter SNPs (Duggan et al., 2006). The lack of consistency between these two studies is unsurprising given the differing nature inflammatory stimuli. As alluded to in the previous chapter, the sterile inflammatory response invoked by exposure to the cardiopulmonary bypass circuit may produce a very different response to the infective inflammatory stimulus observed in septic patients. Additionally, the relatively small numbers recruited to the septic group may preclude meaningful analysis of any association between haplotypes and phenotypes.

Additionally, we did not observe any association between IL-10 genotype and IL-10 mRNA levels. The most commonly described functional association in the IL-10 promoter region is at position -1082 with a G allele associated with higher stimulated IL-10 production (Schaaf et al., 2003; Stanilova et al., 2006). An A allele at position -592 has also been associated with lower levels of IL-10 following stimulation (Stuber, 2003). In each case the high producing allele has been associated with either sepsis susceptibility (Stanilova et al., 2006; Stuber, 2003), the development of septic shock (Schaaf et al., 2003) or an increased mortality in response to a septic insult (Stanilova et al., 2006). An over producing IL-10 allele is unlikely to be of benefit to the host in a septic setting as it may contribute to the detrimental anti-inflammatory phenotype characterised in chapter 3. However, previous functional studies have quantified IL-10 using ELISA based techniques and the reservations around this technique in association with *ex vivo* stimulated cytokine release remains.

The inconsistencies in the published data on IL-10 association studies in sepsis have been highlighted in a recent review (Clark and Baudouin, 2006). A

Bayesian analysis indicated that many of the studies reporting a positive association between genotypes and sepsis were likely to represent false-positive associations. If there is a true relationship between IL-10 genotype and sepsis susceptibility or if the IL-10 SNPs assessed have a functional impact on IL-10 levels then the study presented here is unlikely to be large enough to detect such an effect.

We failed to observe an association between PAI-1 4G/5G variants and any of the clinical correlates assessed. Specifically, presence of the 4G allele was not associated with abnormalities of haemostasis as assessed by the platelet count or the coagulation screen. It has been previously demonstrated using promoter constructs that the 4G allele produces six times as much PAI-1 mRNA as the 5G allele in response to an inflammatory stimulus (Dawson et al., 1993). The PAI-1 gene is of interest in sepsis as activation of the inflammatory and coagulation pathways are closely related and interdependent. Activation of the coagulation pathways are detected in most patients with severe sepsis (Hermans et al., 1999) and administration of endotoxin to healthy volunteers is associated with an increase in tissue PAI-1 production (Suffredini et al., 1989).

Some studies have demonstrated an association between the 4G allele and increased mortality secondary to meningococcal sepsis (Haralambous et al., 2003; Hermans et al., 1999) whereas other studies failed to confirm excess mortality in this setting (Westendorp et al., 1999). However, meningococcal sepsis represents a distinct disease entity and affects primarily a younger population than the cohort presented here. Although coagulation abnormalities are observed in virtually all forms of sepsis, the severity of the coagulopathy is especially prominent in meningococcal sepsis. Therefore extrapolation of these results to a heterogeneous group of septic patients may be misleading. Again, as an association study, this current study is

underpowered to make definitive statements on the likely influence of the PAI-1 polymorphism in septic patients based on the results presented.

8.5 Conclusion

We have described an association between TNFα genetic variants, TNFα mRNA levels and outcome in our cohort of septic patients. Whilst the results contradict some previously published work on functional TNFα polymorphisms they do incorporate the results presented in chapter 2 into a coherent argument for a detrimental anti-inflammatory phenotype in our septic cohort. However, caution must always be advised when interpreting the results of genetic studies from populations of relatively small sizes.

We conclude that genotypic analysis does have a place in risk stratification in sepsis. Genetic variants in the TNFα promoter at positions –863 and –308, or sites in linkage disequilibrium with these variants may influence TNFα production whereas variants at positions -238 and –857 are unlikely to exert a strong effect either individually or as a haplotype. Additionally, functional in vivo studies should take advantage of the increased sensitivity QRT-PCR above ELISA based techniques when assessing the influence of genetic variation on gene function.

Chapter 9 - General discussion

9.1 Advantages of the study design

In this study of the human response to infection, we have described the differences between an efficient, appropriate immune response resulting in the clearance of infection with minimal host disease and an inappropriate immune response resulting in critical illness, organ failure and a high mortality. In contrast to this approach, previous investigators have relied on either serial measurements (Goldie et al., 1995; Headley et al., 1997) or healthy controls (Gogos et al., 2000; Pachot et al., 2005a) as comparator groups. However, by studying a group of patients with infection and no critical illness we can describe an appropriate immunological and inflammatory response to infection *in vivo*, a description that has been lacking from the literature to date. We have then identified, from the differences between these groups, possible causative factors that may render an immune response inefficient or inappropriate.

Critically, the differing responses between the groups do not appear to be related to pre existing illnesses, organ impairment or morbidity status. The stated exclusion criteria ensured that both groups were comparable in terms of underlying illness. It is notable that the group of patients that do not progress to critical illness in response to their infectious insult were significantly older than those patients that did progress to severe sepsis. It is tempting to speculate that the relative longevity observed in these patients may be explained by an inherent ability to tolerate infectious episodes with relative impunity.

9.2 Effector functions and regulation of the distal cytokines

We have demonstrated that by using a combination of the mRNA levels of TNFα, IFNγ and IL-10, it is possible to differentiate the severity of response to, and

outcome following, an infectious insult, with the overall picture implicating an anti-inflammatory phenotype, characterised by a deficiency of TNFα and IFNγ, as being detrimental in the setting of infection. These genes are distal effector cytokines possessing properties that are crucial to an efficient and effective host bactericidal response. Although derived from different cell types, TNFα and IFNγ have synergistic physiological properties, both through the induction of common transcription factors and the also the ability of IFNγ to induce transcription of the TNFα receptor (Boehm et al., 1997). Consequently, it is plausible that a relative deficiency of both TNFα and IFNγ may be particularly detrimental to the host bactericidal response to infection.

The distribution of TNFα and IFNγ mRNA levels across the three groups is particularly interesting. TNFα mRNA levels are raised to a greater extent in the bacteraemic group in comparison to the ICU group. This implies that upregulation of TNFα production may be an appropriate beneficial response to a septic insult. Although this hypothesis may be contrary to earlier theories on the pathophysiology of sepsis (Hotchkiss and Karl, 2003), it does provide a logical and sound mechanistic explaination of the poor results observed in previous trials of anti-TNFα compounds in human sepsis (Abraham et al., 1997; Fisher et al., 1996). In contrast, IFNγ mRNA levels are decreased in the ICU group in comparison to both the bacteraemic and control groups suggesting that this decrease is inappropriate and is unlikely to be of benefit as it is associated with infection and associated critical illness.

The differing patterns that we have observed amongst these proinflammatory cytokines are likely to reflect both the differing cellular origins and the different regulatory pathways of these two classical proinflammatory cytokines. It is plausible that the high IL-10 levels observed in the ICU group directly inhibit production of

IFNγ through inhibition of Th1 cell differentiation and subsequent IFNγ production. Indeed, it has previously been demonstrated that IL-10 directly inhibits IFNγ production from Th1 clones (Bjerre et al., 2004; Fiorentino et al., 1989; Fiorentino et al., 1991). Additionally, direct inhibition via IL-10 may explain, in part, why IL-12 levels only account for a surprisingly small proportion of the variability observed in IFNγ levels.

There is a potential additional novel inhibitory pathway attributable to IL-10 that is worth considering. This pathway is implicated by the association of IL-10 and IκBL mRNA in each of the patient groups. Whilst the exact function of IκBL is as yet undefined it is purported to function in a manner similar to IκBα and therefore may interact with IL-10 and contribute to the production of an anti-inflammatory phenotype (Wang et al., 1995). As discussed in chapter 6, this may plausibly involve either an IL-10 mediated upregulation of the activity of IκBL or alternatively, higher IκBL levels may lead to excess IL-10 production in a manner similar to that demonstrated by the IκB family (Wilson et al., 2005). A reduction in the activity of NFκB, secondary to raised IκBL levels, resulting in a less efficient bactericidal response to infection may, at least partially, explain the increased morbidity and mortality in the ICU group. The intracellular effects of excess TNFα, mediated through exaggerated gene expression, may plausibly be moderated by the lesser NFκB activity through such a mechanism that includes the effects of IκBL.

Notably, IκBL is a gene whose structure has only recently been elucidated and whose exact function is as yet to be defined. Other members of this family, such as IκBz, are currently undergoing preliminary investigations, and it is likely that other members of this family of transcriptional regulators will be uncovered in the future. Therefore, it seems likely that the regulation of the intracellar effects of the distal

effector cytokines that we have investigated will prove to be more complex than is currently appreciated and is likely to involve transcription factors yet to be discovered.

9.3 The complexity of Th17 regulation

Whereas the pattern of cytokine mRNA across the three groups implicates IL-12 and IL-10 in the regulation of IFNγ levels, the same mechanisms do not explain why TNFα levels are raised in the ICU group but are higher again in the bacteraemic group. Interestingly, we describe a strong association between TNFα and IL-23 on day 7 of ICU stay whilst IL-23 levels and the IL-23:IL-27 ratio also distinguishes between the three groups on day 1, with reduced IL-23 associated with a poorer outcome from a septic insult.

The relevance of IL-23 as a principal regulator of the recently defined Th17 response is now appreciated (Weaver et al., 2007). IL-23 has important functions in both innate and adaptive immunity. IL-23 can drive an innate immune response, characterised by IL-17 production from T cells and TNFα production from macrophages, independent of the adaptive immune response. IL-17, in turn, induces TNFα production along with a host of proinflammatory mediators from macrophages. It has been hypothesised that Th17 cells have evolved to provide adaptive immunity specific to particular classes of pathogen. Animal models have demonstrated a critical role for the IL-23/IL-17 axis in pulmonary host defence against *Klebsiella pneumoniae* and intravenous *Candida albicans* infection (Happel et al., 2005; Huang et al., 2004). Similarly, investigators have described differences in the IL-12/IL-23 responses when stimulating with intact gram-positive bacteria and when using purified bacterial products along with whole bacteria (Smits et al., 2004). However,

we did not observe such a difference in regulatory cytokine production in response to different classes of invading microorganisms possibly due to the relatively small numbers recruited and thus represented in each class.

It is plausible that the observed differences in IL-23 mRNA levels between the three groups may also contribute to the relative deficiency in TNFα observed in the ICU group, with IL-23 acting either directly or via a mechanism involving IL-17. However, in order to determine whether alterations in IL-23 concentrations influence the immune response through the Th17 cellular response, it would be necessary to assay IL-17. Nonetheless, the distribution of IL-23 amongst the three groups, coupled with the strong association between IL-23 and TNFα on day 7, implicates the Th17 response as being important in the systemic response to sepsis. This is an important observation as previous work in IBD implicates the Th17 only in the local inflammatory response whilst the IL-12/IFNγ pathway has been shown to be of importance in systemic inflammation (Uhlig et al., 2006).

A model has been proposed for Th17 development, which envisages IL-6 and TGFβ inducing the production of a transcription factor, RORγt, which is essential for Th17 differentiation and necessary for the induction of IL-23 receptor gene expression. Although Th17 cell development is initiated without the presence of IL-23, these cells are not fully committed to the Th17 phenotype at this stage. IL-23 exposure is required to lock these cells into the developmental pathway effecting functional maturation as Th17 cells. This may explain, in part, the lack of association between IL-23 and TNFα on day 1 in the ICU group as other factors may be of greater importance in TNFα regulation at this stage.

Critically, IL-6 acts to deviate TGFβ driven development of Foxp3-expressing T regulatory cells towards Th17 cells (Bettelli et al., 2006). Therefore, it is plausible

that IL-6 levels on day 1 determine TNFα production, mediated in part through elevated IL-17. Whilst we have demonstrated increased IL-6 protein in the ICU group in comparison to healthy controls, it would have been interesting to assess the levels in the bacteraemic group. Higher levels in the bacteraemic group would clearly indicate a protective role for IL-6. In the past, IL-6 has been used as a marker of disease severity in sepsis and we have also demonstrated an association between increased IL-6 and an increased disease severity. Importantly, this association may be reactive as opposed to causal as it may represent the bodies attempt to mount an exuberant Th17 response in the early stages of infection.

However, in chapter 7 we alluded to the potential role of IL-6 in promoting a Th2 response to infection, thereby implicating this cytokine as being involved in regulating and promoting excess IL-10 production and reduced IFNγ production. Theoretically, IL-6 has the ability to function in ways that are immunologically polar opposites and may lead to a pro or anti-inflammatory phenotype. It is unlikely that IL-6 would fulfill both functions simultaneously as this would be self-defeating to a large extent. Therefore, the role of IL-6 in Th1/Th2/Th17 development *in vivo* remains unclear. We have presented two conflicting yet plausible scenarios. In one IL-6 is a Th2 polarising cytokine, contributing to inflammation but inhibiting bactericidal properties of the host and thereby contributing to critical illness. In the other, IL-6 is seen as a Th17 polarising cytokine, contributing to inflammation and pathogen elimination, raised levels of IL-6 being reactive to a septic insult and not a cause of excess mortality. This is again indicative of the complexities involved in the immunological response to a septic insult *in vivo*. Analysis of IL-17 and IL-6 mRNA levels in the three patient groups may go some way towards clarifying the role of IL-6 in human sepsis.

In addition to IL-6 and IL-23, IL-27 is likely to play a role in the Th17 response to an infectious insult and this too may be reflected in the relatively reduced TNFα levels observed in the ICU group. It has previously been demonstrated that IL-27 is involved in suppressing the development of Th17 effectors (Stumhofer et al., 2006). Inappropriately raised IL-27 in a cohort of patients with sepsis may be responsible for stunting a Th17 response, TNFα production and subsequent bactericidal activity. Again, measurement of IL-17 mRNA levels will be required to elucidate the contribution of IL-27 to the inflammatory response to human sepsis.

It is worthwhile recalling the changes that have occurred in the classification of IL-27 since its discovery. Originally labelled as a primarily proinflammatory compound which enhanced T cell responses (Hunter, 2005) it has recently been appreciated that its primary function may be to negatively regulate the development of IL-17 producing Th cells (Batten et al., 2006; Stumhofer et al., 2006). It is, of course, plausible that IL-27 fufils functions both as a suppressor and an enhancer of the Th17 cellular response in differing scenarios. This again serves to reinforce both the complexities of the immune response and the dynamic nature of research into T cell regulation. This complexity is reflected by the inability of any single cytokine measured in our patient cohort to accurately predict patient response to an infectious insult.

9.4 Relevance of the presented data

The data presented here are of particular importance in the understanding of the potential pitfalls associated with immunomodulating therapies. This is particularly the case for researchers seeking to develop novel therapies to treat organ-specific autoimmune pathologies. Initial therapies developed for this purpose were quite non-

specific, targeting distal effector cytokines such as TNFα. However, an increased risk of serious infection often accompanied the use of these blocking agents (Bongartz et al., 2006) leading researchers to develop more targeted and specific agents with the aim of leaving an intact immune response to infection. Trialled therapies now take the form of antibodies to subunits of particular cytokines in order to increase specificity. A clinical trial utilising p40 antibodies has been successful in the treatment of IBD (Mannon et al., 2004). However, antagonists to the common p40 receptor subunit blocks both IL-12 and IL-23 signalling (Fuss et al., 2006) whereas an antagonist to the p19 subunit would be specific for IL-23. This is significant as loss of the IL-12 signalling pathway increases susceptibility to viruses, mycobacterial and salmonella species whereas antagonists of p19 have been lauded as compounds that have the potential to ameliorate inflammation whilst being less likely to leave the host susceptible to such opportunistic pathogens. This hypothesis originates, in part, from animal models of colitis that demonstrates that IL-23 has a role in mucosal immunopathology but not in the systemic immune response (Uhlig et al., 2006). As previously mentioned, the distribution of IL-23 in the three groups of patients implies that IL-23 does play a role in the systemic immune response. As such antagonists to the p19 subunit may leave patients susceptible by blunting the immune response to a systemic infection as in the case of sepsis.

Similarly, it has been suggested that treatment with both IL-27 and agents that block the physiological activities of IL-27 may prove useful in the treatment of inappropriate immune responses. The data presented here should urge caution in the use of supplemental IL-27 in the setting of an overly active immune response as these agents may increase susceptibility to opportunistic infections.

Although the administration of IL-6 antibodies have been shown to be protective in an animal model of sepsis (Starnes et al., 1990), this cytokine may be critical to the initiation of a protective Th17 response in human sepsis. Therefore, the presence of IL-6 may be necessary for optimal host bactericidal functions and IL-6 antibodies may prove to be detrimental in human sepsis. In contrast, it is also plausible that excess IL-6 contributes to a Th2 anti-inflammatory phenotype and that neutralisation of IL-6 may be of benefit.

9.5 Study limitations

Although we have utilised mRNA expression levels as a proxy for estimating functional differences that occur at a protein level the optimal representation of gene function *in vivo* is generally considered to be protein quantification. There are a number of reservations surrounding the use of mRNA as a surrogate of protein levels, such posttranscriptional changes not being accurately identified when assaying mRNA. However, it has also been suggested that the majority of the previously reported lack of correlation observed between mRNA and protein may be explained by the technical imprecision of the methods employed to determine protein expression levels (Hack, 2004). This is particularly problematic when dealing with genes expressed at relatively low levels such that commercially available kits have difficulty detecting protein product. Indeed, we faced this particular problem when assaying TNFα, IL-10 and IFNγ protein product as we found that the ELISA technique we employed was not sufficiently sensitive to detect protein in the *in vivo* situation in the majority of patients assessed.

Recently however, investigators have reproducibly found positive correlations between mRNA and proteins expressed at low levels using isotope coded affinity tags

to quantify protein expression (Fu et al., 2007). These findings imply that perhaps mRNA levels may act as an accurate surrogate of protein expression in the setting of human sepsis as we have demonstrated relatively limited protein production in the *in vivo* situation.

We have made a number of assumptions about the ability of the regulatory cytokines, IL-23 and IL-27, to influence Th-17 cell development thus affecting the host inflammatory response. The optimal method to confirm these assumptions is to assay the $CD4^+$ Th17 cells or alternatively the production of IL-17 mRNA as a surrogate of Th17 activity. Due to the lack of available biological material this will have to be performed in a different patient cohort in future investigations.

Similarly, we have presented two scenarios in which IL-6 may act in opposing fashions as either a Th2 polarising cytokine or as a Th17 polarising cytokine. It is also plausible, given the complexity of the immune response to infection, that both functions are fulfilled at differing time points or in different scenarios. To definitively answer this question IL-6 should be assayed in a bacteraemic group and compared to a critically ill septic group or studied separately in an *in vitro* setting. This will again involve further research.

Finally, adequate patient numbers are of vital importance to accurately identify the effects of most genetic variants. As we have relatively limited numbers of patients to study, it is therefore reasonable to suspect that we may have missed the functional importance of some of the SNPs assayed as a consequence of an inadequately powered study. It is plausible that these genetic variants may have modest effects on transcription or function that would only be recognised in a larger patient population. Therefore, the negative findings reported in chapter 8 should be

repeated using a much larger patient cohort before definitive statements can be made regarding the functional consequences of these SNPs.

9.6 Concluding remarks

We can explain plausibly how the pattern of distal effector cytokines affects the bactericidal host response to a septic insult and we have also hypothesised how this pattern of purported T helper cell development is regulated. Critically however, we lack data to explain the initiating factor leading to a dysregulated immune response to infection. Whilst our results do suggest a genetic contribution to the individual immune response the genetic variability we assessed explains only a minority of the variability in the pattern of cytokine mRNA levels observed. Perhaps future research will help to identify strongly functional SNPs and haplotypes in regulatory genes or their receptors that will then be assessed in the *in vivo* situation of human sepsis. Interestingly, a recent genome wide association study described a highly significant association between the IL-23 receptor and propensity to develop inflammatory bowel disease (Duerr et al., 2006). However, large patient numbers will be required to elucidate the genetic factors contributing to ICU illness. In terms of functional analysis, using the technique of QRT-PCR, as described in this study, will allow sensitive and accurate analysis of gene expression in contrast to the ambiguous results previously seen when using ELISA based techniques.

The central questions driving recent research into sepsis and critical illness are whether the identification of impending critical illness is feasible and whether there is data available to accurately predict outcome. Furthermore, in answering these questions it may be possible to detect points of curative intervention. Whilst the clinical scoring systems provide reasonable data on subsequent outcome, biomarkers have gained in popularity as a method of diagnosing and classifying sepsis. Although some biomarkers such as procalcitonin, IL-6, CRP and lipopolysaccharide binding protein have proved useful in this regard, they tell us little regarding the identification

of potential points of curative intervention when assayed alone (Gaini et al., 2006; Uzzan et al., 2006). In contrast, the approach taken in this study, to assay a number of interacting and functionally linked inflammatory genes permits some clarification of the complex inflammatory signalling cascade that underlies the clinical course of the syndrome.

Taken simplistically, a common thread running through the results is the association of increasing severity of illness and death with alterations in biomarkers that could potentially lead to impaired bactericidal host defences. This consistency of the results is apparent in the case of increased IL-10, IL-27 and ADMA and reduced TNFα, IFNγ and IL-23. These data are potentially of use in developing therapies for the treatment of patients with severe sepsis. Therapies may take the form of the administration of either recombinant factors known to be deficient in these patients or of blocking compounds to factors demonstrated to be produced in excess that may also impair host defences. The probability of developing the much-anticipated "magic bullet" in these patients is highly unlikely given the heterogenous nature of both the disease and the patient group and the enormous complexity of the immune response to infection in humans. However, this research introduces the possibility of using techniques such as QRT-PCR to identify patients with a dysregulated immune response to infection and anticipates the treatment of these patients in a timely and targeted fashion.

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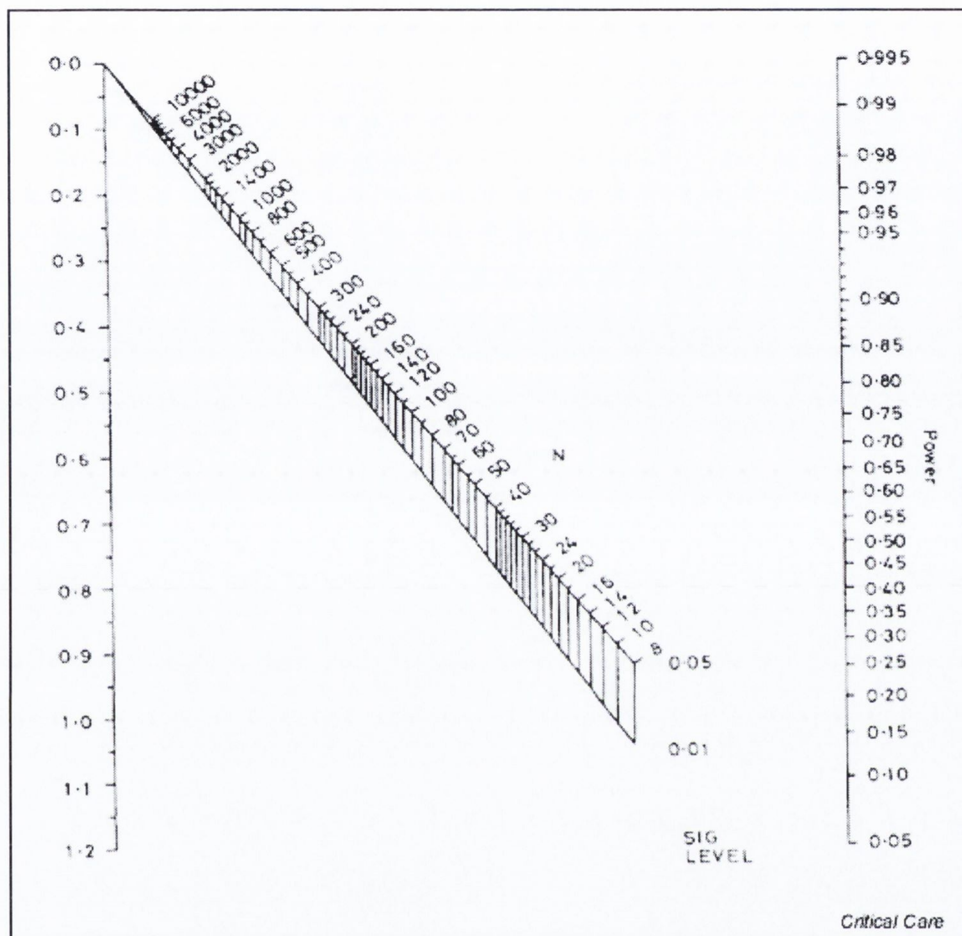
Appendix

A.1: A nomogram used for the calculation of sample size(Whitley and Ball, 2002)



In order to calculate sample size a straight line is drawn connecting the required power to the calculated standardised difference. Where this line intersects the diagonal line marked 0.05 is the sample size for an α of 0.05.

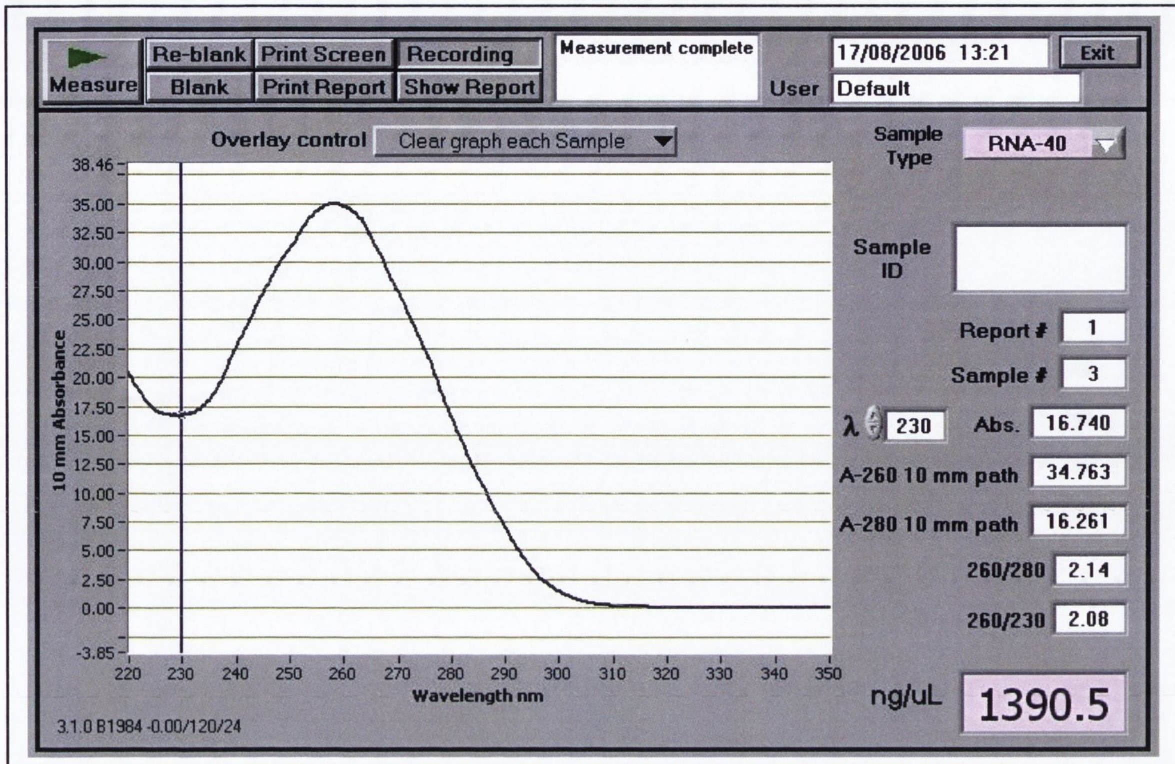
A.2: Oligonucleotides used for standard preparation

mRNA targets	Oligonucleotides (5′→3′)[a]	Product size (bp)	Conditions for "classical" PCR[b]
IL10	F296: TTTACCTGGAGAGGTGATG R771: TTGGGCTTCTTTCTAAATCGT	476	A=56, Mg=1.5
TNFα	F83: ACCATGAGCACTGAAAGCAT R488: AGATGAGGTACAGGCCCTCT	406	A=58, Mg=1.5
IFNγ	F154: TTGGGTTCTCTTGGCTGTTA R632: AAATATTGCAGGCAGGACAA	479	A=58, Mg=1.5
B-actin	F745: CCCTGGAGAAGAGCTACGA R1253: TAAAGCCATGCCAATCTCAT	509	A=58, Mg=1.5
IL12	F185: AGCCTCCTCCTTGTGGCTA R412: TGTGCTGGTTTTATCTTTTGTG	228	A=58, Mg=1.5
IL18	F133: AGTCTACACAGCTTCGGGAAGA R653: GTCCTGGGACACTTCTCTGAAA	113	A=60, Mg=1.5
IL4	F27: TAATTGCCTCACATTGTCACT R52P: ATTCAGCTCGAACACTTTGAA	503	A=58,Mg=1.5

[a] F and R indicate forward and reverse primers, respectively; numbers indicate the sequence position.

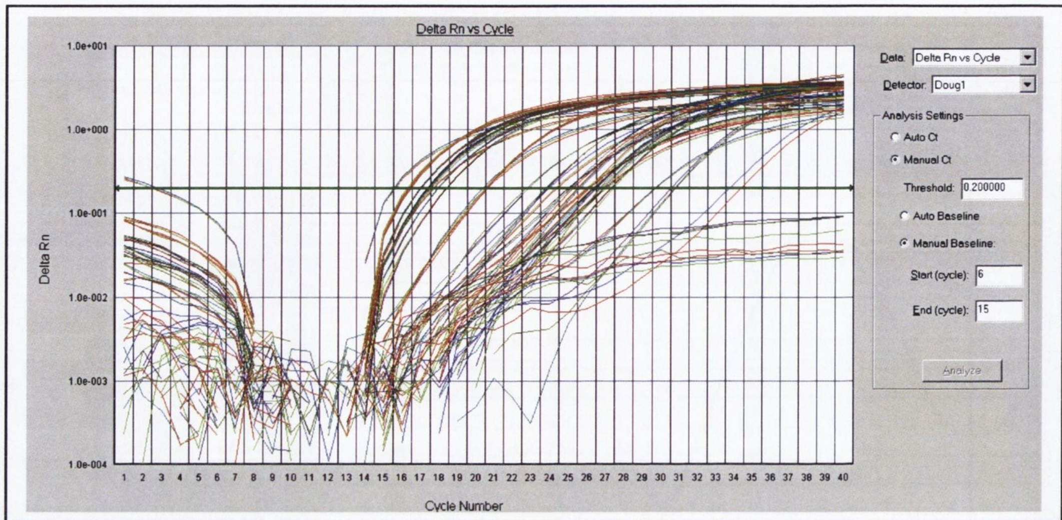
[b] Conditions, for all targets, were as follows: denaturation at 95°C for 20s, annealing (temperature as stated (A)) for 20s and elongation at 72°C for 45s, for a total of 35 cycles. MgCl2 concentration (Mg,mM) was as stated.(For the complete procedure see Stordeur et al., 1995, PCR for IFNγ(Stordeur et al., 1995).

A.3: Nanodrop ND 1000 UV-Vis spectrophotometer



The nanodrop allows saving in the use of biological material (1µl Vs 10µl with the spectrophotometer). The displayed curve also supplies better information about the quality of the sample as opposed to just receiving the A_{260}:A_{280} ratio as is the case with the spectrophotometer.

A.4: ABI Prism 7700



Fluorescence is depicted on the Y axis with cycle number on the X axis. The background fluorescence is depicted by the horizontal green line. Two different genes are analysed here with the housekeeping gene breaching background fluorescence sooner than the target gene. The non-template controls fail to breach the background fluorescence.

A.5: Oligonucleotides for real-time PCR

mRNA targets	Oligonucleotides (5′→3′)[a]	Product size (bp)	Final concentration (nM)[b]
B-actin	F976: GGATGCAGAAGGAGATCACTG	90	F300; R300
	R1065: CGATCCACACGGAGTACTTG		
	P997: 6Fam-CCCTGGCACCCAGCACAATG-Tamra-p		
IFN-γ	F464: CTAATTATTCGGTAACTGACTTGA	75	F600; R900
	R538: ACAGTTCAGCCATCACTTGGA		
	P491: 6Fam-TCCAACGCAAAGCAATACATGAAC-Tamra-p		
IL12p35	F212: CTCCTGGACCACCTCAGTTTG	76	F600; R900
	R287: GGTGAAGGCATGGGAACATT		
	P234: 6Fam-CCAGAAACCTCCCCGTGGCCA-Tamra-p		
IL18	F151: AAGAGGAAAGGAACCTCAGACC	113	F300; R600
	R263: CCACAAAGTTGATGCAATTGTC		
	P175: 6Fam-CCAGATCGCTTCCTCTCGCAACA-Tamra-p		
IL4	F174: ACTTTGAACAGCCTCACAGAG	74	F300; R900
	R247: TTGGAGGCAGCAAAGATGTC		
	P204: 6Fam-CTGTGCACCGAGTTGACCGTA-Tamra-p		
IL23p19	F533: TACTGGGCCTCAGCCAACT	117	F900; R900
	R649: GAAGGATTTTGAAGCGGAGAA		
	P597: 6Fam-CCTCAGTCCCAGCCAGCCATG-Tamra-p		
TNFα	Supplied as a precustomised mix from Applied Biosystems		F900; R900; P250
IL10	Supplied as a precustomised mix from Applied Biosystems		F900; R900; P250
IKBL	Supplied as a precustomised mix from Applied Biosystems		F900; R900; P250
IL27p28	Supplied as a precustomised mix from Applied Biosystems		F900; R900; P250

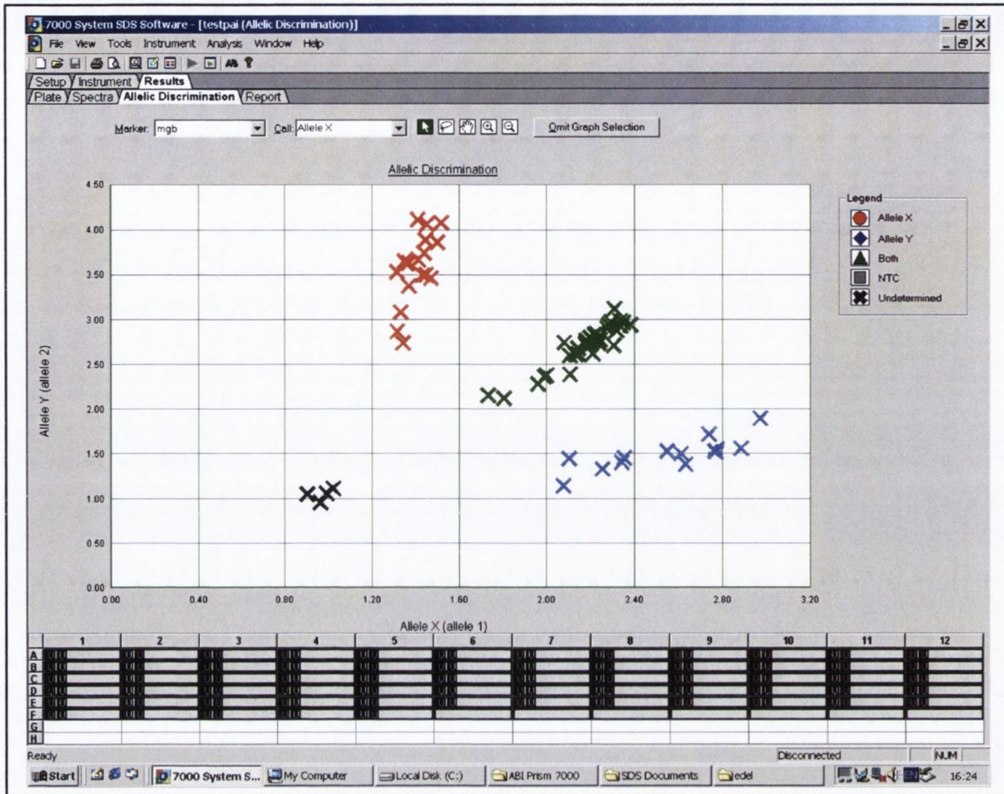
[a] F, R and P indicated forward and reverse primers and probes, respectively; numbers indicated the sequence position.
[b] Final concentration of forward (F) and reverse (R) primers.

A.6: Set-up for a 96 well plate for QRT-PCR quantifying TNFα

TNF 10^6	TNF 10^6	TNF 10^6	TNF 10^5	TNF 10^5	TNF 10^5	TNF 10^4	TNF 10^4	TNF 10^4	TNF 10^3	TNF 10^3	TNF 10^3
TNF 10^2	TNF 10^2	TNF 10^2	Bactin 10^9	Bactin 10^9	Bactin 10^9	Bactin 10^8	Bactin 10^8	Bactin 10^8	Bactin 10^7	Bactin 10^7	Bactin 10^7
Bactin 10^6	Bactin 10^6	Bactin 10^6	Bactin 10^5	Bactin 10^5	Bactin 10^5	Pt 1 TNF	Pt 1 TNF	Pt 1 TNF	Pt 2 TNF	Pt 2 TNF	Pt 2 TNF
Pt 3 TNF	Pt 3 TNF	Pt 3 TNF	Pt 4 TNF	Pt 4 TNF	Pt 4 TNF	Pt 5 TNF	Pt 5 TNF	Pt 5 TNF	Pt 6 TNF	Pt 6 TNF	Pt 6 TNF
Pt 7 TNF	Pt 7 TNF	Pt 7 TNF	Pt 8 TNF	Pt 8 TNF	Pt 8 TNF	Pt 9 TNF	Pt 9 TNF	Pt 9 TNF	Pt 10 TNF	Pt 10 TNF	Pt 10 TNF
Pt 11 TNF	Pt 11 TNF	Pt 11 TNF	Pt 12 TNF	Pt 12 TNF	Pt 12 TNF	Pt 13 TNF	Pt 13 TNF	Pt 13 TNF	Pt 14 TNF	Pt 14 TNF	Pt 14 TNF
Pt 1 Bactin	Pt 1 Bactin	Pt 1 Bactin	Pt 2 Bactin	Pt 2 Bactin	Pt 2 Bactin	Pt 3 Bactin	Pt 3 Bactin	Pt 3 Bactin	Pt 4 Bactin	Pt 4 Bactin	Pt 4 Bactin
Pt 5 Bactin	Pt 5 Bactin	Pt 5 Bactin	Pt 6 Bactin	Pt 6 Bactin	Pt 6 Bactin	Pt 7 Bactin	Pt 7 Bactin	Pt 7 Bactin	Pt 8 Bactin	Pt 8 Bactin	Pt 8 Bactin
Pt 9 Bactin	Pt 9 Bactin	Pt 9 Bactin	Pt 10 Bactin	Pt 10 Bactin	Pt 10 Bactin	Pt 11 Bactin	Pt 11 Bactin	Pt 11 Bactin	Pt 12 Bactin	Pt 12 Bactin	Pt 12 Bactin
Pt 13 Bactin	Pt 13 Bactin	Pt 13 Bactin	Pt 14 Bactin	Pt 14 Bactin	Pt 14 Bactin	NTC TNF	NTC TNF	NTC TNF	NTC Bactin	NTC Bactin	NTC Bactin

Dilution series for TNFα and β Actin are included on the plate with the copy numbers per μL indicated on the table. NTCs are constructed using both TNFα and β Actin primers and probes separately as indicated. Pt indicates patient number.

A.7: Scatterplot generated from the ABI prism 7000 for allelic discrimination



A clear distinction can be observed between 4G homozygotes which are depicted in red, 5G homozygotes in blue, heterozygotes in green with non template controls in black.

A.8: Primers, probes, PCR reagent mix and reaction conditions for Taqman genotyping

Forward Primer 5'-AGCCAGACAAGGTTGT TGACA-3'

Reverse Primer 5' GCCGCCTCCGATGATACAC-3'

Allele 1 Probe 5'- CTGACT**CCCC**ACGTGT-3', *5'Fluor Label, 6-FAM*

Allele 2 Probe 5' – CTGACT**CCCCC**ACGTGT-3' *5-Fluro Label, VIC*

PCR amplification was then performed using:

- 12.5μL of Taqman universal PCR mastermix (Applied Biosystems/ Perkin Elmer), containing AmpliTaq Gold DNA polymerase.
- 0.2μM of the probe labelled with the FAM dye,
- 0.2μM of the probe labelled with the VIC dye,
- 0.9μM of the forward primer,
- 0.9 μM of the reverse primer and
- 9.875μL of water containing 2ng genomic DNA.

The cycling conditions consisted of a denaturation step at 95° C for 10 minutes, 40 cycles at 92° C for 15 seconds, a 60 second annealing step for 40 cycles at 62° C and finally a holding temperature of 15° C.

A9: Related publication. The occurrence of severe sepsis and septic shock are related to distinct patterns of cytokine gene expression. *Shock* 2006 Dec;26 (6):544-50.

A10: Related publication. Septic shock is correlated with asymmetrical dimethyl arginine levels, which may be influenced by a polymorphism in the dimethylarginine dimethylaminohydralase 2 gene: a prospective observational study. ***Critical Care*** **2006; 10(5):R139.**

A11: Related publication. The human response to infection is associated with distinct patterns of interleukin 23 and interleukin 27 gene expression. *Intensive Care Medicine* 2008 Apr; 34(4):683-691.

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THE OCCURRENCE OF SEVERE SEPSIS AND SEPTIC SHOCK ARE RELATED TO DISTINCT PATTERNS OF CYTOKINE GENE EXPRESSION

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ABSTRACT—Patient response to acute bacterial infection is highly variable. Differing outcomes in this setting may be related to variations in the immune response to an infectious insult. Using quantitative real-time polymerase chain reaction, we quantified gene expression of the tumor necrosis factor α(*TNFα*), interferon γ (*IFNγ*), and interleukin 10 (*IL10*), *IL12p35*, and *IL4* genes in 3 patient groups. These groups consisted of an intensive care unit (ICU) cohort who presented with severe sepsis or septic shock, a group of noncritically ill ward patients with documented Gram-negative bacteremia, and a group of healthy controls. Greater interleukin 10 messenger RNA (mRNA) levels were detected in the ICU group in comparison with both the bacteremic and control groups ($P < 0.0001$). More TNF-α mRNA was detected in the ICU group when compared with the control group ($P < 0.0001$). However, TNF-α mRNA was most abundant in the bacteremic group ($P = 0.0007$). Lesser IFN-γ mRNA levels were detected in the ICU group when compared with both the bacteremic and control groups ($P < 0.0003$). Cytokine mRNA levels were not associated with the occurrence of shock upon admission to ICU. On the seventh day of ICU stay, the presence of shock was associated with lesser IFN-γ mRNA ($P = 0.0004$) and lesser TNF-α mRNA ($P = 0.001$). Survivors had greater TNF-α mRNA copy numbers on day 7 of ICU stay than nonsurvivors ($P = 0.002$). We conclude that a proinflammatory response is the appropriate response in the setting of infection and is associated with lesser requirements for inotropes and lesser mortality. Quantitative real-time polymerase chain reaction can be used to predict infection outcome in clinically relevant situations where enzyme-linked immunosorbent assay testing has proved disappointing.

KEYWORDS—Sepsis syndrome, cytokines, interferon type II, TNF-α, RT-PCR

INTRODUCTION

Acute bacterial infection has a whole gamut of presentation, ranging in severity from a mild self-limiting illness to an overwhelming insult with resultant multiple organ failure. This latter "sepsis syndrome" (1) is a devastating illness and is a common intensive care unit (ICU) admission diagnosis, with an incidence of 3 per 1000 population per annum (2). It has been characterized as a "dysregulation of inflammation" in response to infection, with life-threatening organ failure attributed to a combination of excessive cytokine-mediated inflammation, disseminated coagulopathy, and disruption of the integrity of microvascular endothelium (3). However, in contrast to this hypothesis, a marked immune paresis, often characterized as a predominant anti-inflammatory cytokine response (4, 5), or a deficit in the major histocompatibility complex gene expression (6, 7) is well documented in patients with severe sepsis, with the severity of the immune paresis related to adverse outcome.

These hypotheses, which have been generated primarily by direct measurement of cytokine proteins in blood (5) or in cells stimulated with endotoxin *in vitro* (4, 6), are clearly discordant. In addition, neither of these methods provides a clinically relevant reproducible assay.

However, recent developments in polymerase chain reaction (PCR) technology has provided a sensitive and reproducible assay of cytokine gene expression suitable for use in unstimulated cells, which quantifies absolute levels of cytokine messenger RNA (mRNA), and is termed quantitative real-time PCR (QRT-PCR) (8).

Although this technique has recently been used in patients with septic shock (7, 9), it remains unclear whether there is a distinct pattern of cytokine mRNA production associated with the occurrence of shock in patients with severe sepsis and whether this pattern is consistent or changes over the time course of the disease. Furthermore, the occurrence of severe sepsis in response to bacterial infection may be associated with a pattern of cytokine gene expression, which is distinct from that observed in patients who do not develop critical illness in response to infection. Therefore, QRT-PCR may be useful in predicting the outcome of an infectious insult.

This study was performed, using QRT-PCR, to determine the pattern of cytokine gene expression associated with both severe sepsis and infection in the absence of critical illness and also to determine whether there was a characteristic cytokine pattern linked to the occurrence and resolution of septic shock.

MATERIALS AND METHODS

Patients

This study was conducted in St James's Hospital, Dublin, Ireland and was approved by the institutional ethics committee. Informed written consent was obtained from each patient or a relative. A total of 62 consecutive patients presenting with severe sepsis or septic shock, as defined by the American College

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of Chest Physicians/Society of Critical Care Medicine Consensus Conference (1), as a primary admission diagnosis were enrolled for 12 months. All ICU patients received similar standardized care.

Severity of illness was characterized upon admission to ICU using the Simplified Acute Physiology Score (SAPS II) (10) and the sequential organ failure assessment (SOFA) (11) scoring systems and again, on day 7, with the SOFA score.

Individual clinical and laboratory variables were collected on days 1 and 7 of ICU stay. The recorded variables represented the most significant derangements from normal values recorded for each 24-h period. The source of infection necessitating the ICU admission, subsequent infections over the course of the ICU stay, and the respective pathogenic organisms were noted. Intensive care unit death or survival to ICU discharge was recorded.

Ten consecutive patients, from hospital wards, with a documented Gram-negative bacteremia confirmed on blood culture were identified by the microbiology department and enrolled if no organ failure or impending septic crisis was identified.

Thirteen healthy staff members served as a control group.

Exclusion criteria

Exclusion criteria included the following: (1) pre-existing overt organ failure, (2) infection with the human immunodeficiency virus, (3) patients with neutropenia as a result of chemotherapy, (4) patients receiving long-term treatment with corticosteroids, (5) patients with trauma and burns, and (6) patients with non-Irish white ethnic background.

Blood sampling

Blood sampling was carried out within the first 24 h of ICU admission and again 7 days later. In bacteremic patients, blood sampling was carried out within 24 h of the positive blood culture being reported. Blood samples were collected from healthy controls at 1 time point.

Peripheral blood mononuclear cells (PBMCs) were immediately purified by density gradient centrifugation of EDTA-anticoagulated blood using lymphoprep (Nycomed Pharma, Oslo, Norway).

Serum was obtained from whole blood clotted for 30 min.

Total RNA extraction and reverse transcription

Total RNA was isolated from lysed PBMC using a commercially available kit (Qiagen) following the manufacturer's instructions. To avoid amplification of contaminating genomic DNA, all samples were treated with ribonuclease-free deoxyribonuclease (Qiagen, Hilden, Germany) for 15 min. The quantity and purity of extracted RNA was measured with a spectrophotometer (Eppendorf BioPhotometer; Eppendorf AG, Hamburg, Germany). The quality of the extracted mRNA was verified on an Agilent 2100 Bioanalyzer using the RNA Nano LabChip kit (Agilent Technologies, Palo Alto, Calif).

Total RNA was then reverse transcribed as follows: 11.15 μL of water containing 500 ng of total RNA was first incubated at 65°C for 10 min; 18.85 μL of the reverse transcription mix containing the following components were added: (1) 3 μL of 0.1 mol/L dithiothreitol, (2) 4.5 μL of dimethyl sulfoxide, (3) 2 μL of 100 μmol/L random primers (Invitrogen Corporation, Carlsbad, Calif), (4) 1.25 μL of Moloney murine leukemia virus reverse transcriptase (Invitrogen Corporation), (5) 6 μL 5X first-strand buffer (Invitrogen Corporation), (6) 1.5 μL of 4 mmol/L deoxynucleotide triphosphate mix (Promega Corporation, Madison, Wis), and (7) 0.6 μL of ribonuclease inhibitor (Promega Corporation) 10 U/μL. The samples were then incubated at 37°C for 1 h.

Primers and probes

All primers and probes used in this study were synthesized at Applied Biosystems (Foster City, Calif). Tumor necrosis factor (TNF) α and interleukin (IL) 10 primers and probes were obtained as a precustomized mix (assay ID for TNF-α is Hs00174128_m1, and that for IL-10 is Hs00174086_m1). The β-actin, IL-12P35, and IL-4, and interferon (IFN) γ primers and probes were designed and customized (sequences listed in Appendix 1), as per Stordeur et al. (8).

Real-time PCR

The PCR reactions were carried out in an ABI Prism 7000 (Applied Biosytems). All reactions were performed either in triplicate or in duplicate. Thermocycling was carried out in a 20-μL final volume containing the following: (1) water up to 20 μL; (2) 10 μL of Master Mix (Applied Biosystems); (3) 1, 2, or 3 μL of 6 pmol/μL forward and reverse primers (final concentration of 300, 600, or 900 nmol/L; see Appendix 1); (4) 1 μL of 4 pmol/μL Taqman probe (final concentration of 200 nmol/L) or 1 μL of precustomized primer/probe mix with default primer and probe concentrations (Appendix 1); (5) 0.8 μL of standard dilution or 2.4 μL of complementary DNA (cDNA). After an initial denaturation step at 95°C for 10 min, temperature cycling was initiated. Each cycle consisted of 95°C for 15 s and 60°C for 60 s, the fluorescence being read at the end of this second step. In total, 40 cycles were performed.

Standard curves and expression of the results

The DNA standards for TNF-α, IL-10, β-actin, IL-12p35, IL-4, and IFN-γ consisted of a cloned PCR product that included the quantified amplicon prepared by PCR from a cDNA population containing the target mRNA. Detailed information on these standards is given in Appendix 2. To quantify transcript levels, a standard curve was constructed, for each PCR run, for each selected mRNA target from serial dilutions of the relevant standard. All standard curves showed correlation coefficients of greater than 0.99, indicating a precise log-linear relationship.

The mean efficiency of the standard curves for all target cDNA was 98.75% ± 4.4%. The mRNA copy numbers were then calculated for each patient sample using the standard curve to convert the obtained crossing threshold value into mRNA copy numbers. Results were then expressed in absolute copy numbers after normalization against β-actin mRNA (mRNA copy numbers of cytokine mRNA per 10 million β-actin mRNA copy numbers).

Cytokine enzyme-linked immunosorbent assay

Serum TNF-α, IL-10, IFN-γ, and IL-6 concentrations were measured by enzyme-linked immunosorbent assay (R&D systems, Minneapolis, Minn), following the manufacturers instructions. The lower limit of detection for TNF-α, IL-10, IFN-γ, and IL-6 was 16, 47, 16, and 9 pg/mL. All samples were tested in duplicate.

Statistical analysis

The Wilcoxon rank sum test was used to analyze the differences between groups for continuous variables. Categorical variables were analyzed by chi-square test and Fisher exact test as appropriate. Hierarchical cluster analysis was performed with standardized data using Ward method. Data analysis was performed using the JMP statistical package (SAS Institute, Cary, NC).

RESULTS

After giving their consent, 62 ICU patients, 10 bacteremic ward patients, and 13 healthy controls were recruited into the study. Blood samples were available for PCR analysis from 52 of the ICU patients on the first day of critical illness and from 49 ICU patients on the seventh day of critical illness. A total of 39 ICU patients had blood samples available for PCR analysis at both time points.

The demographics, illness severity scores, and sites of infection for the septic shock and severe sepsis groups on day 1 and the bacteremic group are listed in Table 1. There were

TABLE 1. **Demographics of shocked, nonshocked, and bacteremic groups**

	Shocked	Nonshocked	Bacteremic	*P*
n	42	20	10	
Male	26 (61%)	10 (50%)	4 (40%)	NS
Age, year	70 (56–77)	62 (43–72)	82 (62–85)	0.017
SAPS II score	47 (39–59)	35 (28–41)	27 (21–33)	<0.0001
SOFA score	9 (8–12)	4 (3–5)	0.5 (0–1)	<0.0001
Duration of ventilation, d	9 (3–21)	2 (0–17)		0.049
Duration of ICU stay, d	11 (4–25)	5 (2–22)		NS
Site of infection				
Respiratory	21 (50%)	11 (55%)	4 (40%)	NS
Abdominal	14 (33%)	7 (35%)	3 (30%)	NS
Other	8 (19%)	2 (10%)	3 (30%)	NS

Results are presented for the 3 groups (patients with septic shock and severe sepsis on day 1 and patients with bacteremia). Values are either absolute counts, with percentages or median values with interquartile range in parentheses. Significant differences among the 3 groups were determined by a Kruskal-Wallis test.
NS indicates nonsignificant.

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Table 2. **Demographics of ICU patients**

	Survivors	Nonsurvivors	P
n	45	17	
Male	24 (53%)	10 (59%)	NS
Age, year	62 (19–81)	67 (39–86)	NS
SOFA at day 1	7 (4–10)	9 (9–13)	0.003
SAPS II	40 (33–51)	49 (41–60)	0.02
Ventilated at day 1	36 (80%)	17 (100%)	NS
Shock at day 1	26 (58%)	16 (94%)	0.006
ICU stay	12 (3.5–27)	5 (3–10.5)	0.03
Site of infection			
Respiratory	22 (49%)	10 (59%)	NS
Abdominal	15 (33%)	5 (29%)	NS
Others	8 (18%)	2 (12%)	NS

Results are presented for survivors and nonsurvivors from the ICU group. Age and severity of illness scores are presented as medians, with the interquartile range in parentheses.
Abbreviation is explained in the footnote to Table 1.

significant differences between the groups in the organ severity scores ($P < 0.0001$) and age. Shocked patients on day 1 also had a longer duration of ventilation than non-shocked patients.

The demographics, illness severity scores, and sites of infection for survivors and nonsurvivors in the group with severe sepsis are listed in Table 2. Survivors had lesser illness severity scores and were less likely to be shocked on day 1 compared with nonsurvivors.

Day 1 mRNA comparisons

On the first day of critical illness, TNF-α mRNA levels in the ICU group were greater than those in the control group but less than those in the bacteremic group. IL-10 mRNA levels in the ICU group were greater than those in the control and bacteremic groups. IFN-γ mRNA levels in the ICU group were lesser than those in the control and bacteremic groups. IL-4 mRNA levels in the ICU group were lesser than those in the control and bacteremic groups. IL-12 mRNA levels in the ICU group were less than those in the bacteremic group but similar to those in the control group (Table 3).

Table 3. **Cytokine mRNA quantification in ICU patients with sepsis on day 1 compared with controls and patients with bacteremia**

	ICU day 1	Bacteremia	Control
n	52	10	13
TNF-α	19,866 (11,545–28,270)	36,294 (28,858–72,275)	577 (422–1,679)
P		0.0007	<0.0001
IL-10	851 (573–1,638)	163 (88–363)	66 (33–81)
P		<0.0001	<0.0001
IL-12	2170 (923–4,039)	3,843 (1,937–5,910)	2,123 (1,676–5,597)
P		0.03	NS
IFN-γ	131 (76–350)	745 (285–941)	883 (617–1,216)
P		0.0003	<0.0001
IL-4	9 (2–28); n = 39	201 (22–512); n = 9	161 (58–262); n = 12
P		0.005	0.0004

Results are expressed as mRNA copy numbers per 10 million β-actin mRNA copies.
All values are median and interquartile range. All comparisons are by Wilcoxon rank sum test, with P values stated as uncorrected values. All P values are for comparison with the ICU group.
Abbreviation is explained in the footnote to Table 1.

Multivariate group comparison

A multivariate logistic regression model, which included data for TNF-α, IFN-γ, and IL-10, of patients in the bacteremic and ICU groups was constructed. This model had an R^2 value of 0.63, with a receiver operating characteristic curve value of 0.96. From this model, the respective probabilities belonging to the bacteremic and ICU groups were determined for each patient, and the ratio of these probabilities was considered as a relative risk for outcome in response to infection. This relative risk scoring system could be used to identify the bacteremic group with 100% specificity and 70% sensitivity or identify the ICU group with 100% specificity and 94% sensitivity.

Shock and mRNA levels in the ICU group

In the ICU group, there was no association between the cytokine mRNA levels and the presence or absence of shock on day 1 of critical illness. However, on the seventh day, patients with shock displayed significantly lower TNF-α and IFN-γ mRNA levels compared with the patients without shock (Table 4).

Mortality and mRNA levels

Seventeen (27%) of the 62 ICU patients died during the course of their ICU stay. Of the 52 patients that were analyzed for mRNA levels on the first day of critical illness, 13 died before ICU discharge. There was a nonsignificant trend toward greater IL-10 mRNA levels (1,262 [721–3,169] vs. 776 [539–1,353]; $P = 0.1$; all values expressed as median with interquartile range in parentheses and units as mRNA copy numbers per 10 million β-actin copy numbers) and

Table 4. **The relationship between cytokine mRNA levels and the presence of shock on day 7 in the ICU group**

Cytokine	Shock group	Group without shock	P
n	15	34	
TNF-α	9,667 (6,207–17,291)	19,179 (10,902–28,543)	0.001
IL-10	355 (154–452)	189 (56–312)	0.11
IL-12	2,619 (2,109–8,014)	4,619 (2,669–6,699)	NS
IFN-γ	150 (70–195)	368 (196–772)	0.0004
IL-4	229 (26–671); n = 15	66 (15–270); n = 31	NS

Results are expressed as mRNA copy numbers per 10 million β-actin mRNA copies.
All comparisons are by Wilcoxon rank sum test. All values are stated as median with interquartile ranges.
Abbreviation is explained in the footnote to Table 1.

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lesser IFN-γ mRNA levels (112 [70–140] vs. 180 [76–411]; $P = 0.07$) in nonsurvivors when compared with survivors. However, when ICU patients were dichotomized between those in the highest quartile of IFN-γ mRNA production on day 1 and the remainder, no deaths occurred in the highest quartile group, whereas 13 of the other 39 patients died ($P = 0.02$).

Eight of the 49 ICU patients who had blood samples analyzed for mRNA production after 7 days of critical illness died before ICU discharge. These 8 nonsurvivors had lesser TNF-α mRNA levels (Table 5).

White blood cell count and mRNA levels

The ICU and bacteremic groups were analyzed for an association between white blood cell counts and cytokine mRNA levels for each cytokine. When a correction for multiple testing was used, no association was observed between total white blood cell count or any white cell subset and any of the cytokine mRNAs in either ICU or bacteremic groups at all time points.

Protein levels

Most patients had no detectable TNF-α, IL-10, or IFN-γ protein, and levels were unrelated to mRNA copy numbers for each constituent cytokine. However, IL-6 protein was present in measurable quantities on day 1 (306 [131–731]; all values expressed as median, with interquartile range in parenthesis and units as pg/mL) and day 7 (63 [3.6–174]). On day 1, IL-6 levels discriminated between subsequent survivors and nonsurvivors, levels being higher in nonsurvivors (783 [566–926] vs. 271 [49–470]). Patients who were shocked on day 7 had higher IL-6 levels than those who were not shocked (173 [48–617] vs. 23 [0–117]).

Cluster analysis in the ICU group

A cluster analysis (JMP software), which included ICU data for day 7 TNF-α and IFN-γ mRNA levels, was performed, and a cluster of ICU patients with reduced TNF-α and IFN-γ mRNA levels was characterized. Patients in this cluster were more likely to be shocked on day 7 of critical illness, had greater SOFA scores, and had a significantly higher mortality rate (Table 6).

TABLE 5. **Relationship between cytokine mRNA levels in the ICU group on day 7 of critical illness and subsequent outcome**

	Survivors	Nonsurvivors	*P*
n	41	8	
TNF-α	17,329 (10,766–25,600)	6,898 (3,814–9,605)	0.002
IL-10	255 (133–402)	40 (24–348)	0.08
IL-12	4,672 (2,619–6,885)	2,420 (1,588–6,338)	NS
IFN-γ	293 (158–677)	148 (59–565)	NS
IL-4	76 (27–281); n = 38	178 (10–954); n = 8	NS

Results are expressed as mRNA copy numbers per 10 million β-actin mRNA copies.
All values are median and interquartile range. All comparisons are by Wilcoxon rank sum test, with *P* values stated as uncorrected values.
Abbreviation is explained in the footnote to Table 1.

TABLE 6. **Cluster analysis of TNF- and IFN- mRNA levels in the ICU group on day 7 of critical illness**

Cluster	1	2	*P*
n	31	18	
TNF-α	21,379 (16,218–28,840)	6,760 (2,884–10,715)	0.0001
IFN-γ	407 (190–794)	158 (93–229)	0.0004
Shock	4 (13%)	11 (61%)	0.0009
SOFA	3 (0–6)	10 (8–13)	0.0001
Outcome (death)	2 (6.5%)	6 (33%)	0.04

Results are expressed as mRNA copy numbers per 10 million β-actin mRNA copies.
All continuous variables are quoted as median with interquartile ranges.
All comparisons are by Wilcoxon rank sum test.

DISCUSSION

In this study, patient response to infection was associated with distinct patterns of cytokine mRNA production. More IFN-γ and TNF-α mRNA was detected in the bacteremia group when compared with the ICU group. Lesser IFN-γ and TNF-α gene expression was associated with both the occurrence of severe sepsis in response to infection and the poor outcome in these ICU patients.

IL-10, a potent anti-inflammatory cytokine and a direct inhibitor of macrophage function, exerts anti-inflammatory properties *via* inhibition of nuclear factor κB nuclear translocation, thereby inhibiting proinflammatory cytokine gene expression (12). IL-10 mRNA levels were greatest in patients with sepsis and lowest in patients with bacteremia who did not develop critical illness. This pattern of IL-10 gene expression may account for the relatively deficient IFN-γ production in patients with sepsis because IFN-γ gene expression in antigen-presenting cells may be inhibited by IL-10 (13). Furthermore, exogenous IL-10 is reported to inhibit IFN-γ production in isolated monocytes from patients with sepsis (14).

Because IFN-γ is the signature cytokine for the CD4$^+$ T helper cell (T_H) 1 cytokine response, and IL-10 is a T_H2 cytokine, these data suggest that both the occurrence of severe sepsis and outcome after severe sepsis may be linked to an imbalance in cytokine production in response to infection, with a dominant T_H1 profile providing the optimal defense against infection.

In addition, patients who developed severe sepsis had TNF-α mRNA levels intermediate between that observed in the control and bacteremic groups. This exaggerated TNF-α response in patients with infection who did not develop critical illness may represent an appropriate and protective response. The concept that certain proinflammatory cytokines are beneficial to patients with infection may account for the adverse outcome observed in prior studies of TNF-α antagonists in patients with septic shock (15–17). Furthermore, such a protective role of TNF-α in sepsis may, in part, account for the association between persistent shock and death that we

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observed in conjunction with lesser TNF-α mRNA levels in ICU patients with severe sepsis.

As with TNF-α, IFN-γ possesses proinflammatory properties. The IFN-γ is a pleiotropic cytokine, which augments phagocytic bactericidal activity (18); consequently, the lower IFN-γ mRNA levels observed in patients with severe sepsis and persistent shock is not likely to be a beneficial response. In a fashion similar to the pattern of TNF-α gene expression, the relatively greater levels of IFN-γ mRNA observed in bacteremic patients, in comparison to the ICU patients, may represent an appropriate and beneficial T_H1 cytokine response. This concept is consistent with animal models of sepsis, which confirms the importance of IFN-γ in bacterial clearance (19–21).

In humans, several case series document the beneficial use of exogenous IFN-γ in leishmaniasis, leprosy, and multidrug-resistant tuberculosis (22–24). The therapeutic use of IFN-γ in patients with sepsis has been less extensively reported. To date, 2 studies failed to show any survival benefit from therapy with subcutaneous IFN-γ in patient groups prone to develop sepsis (25, 26). However, a case series of patients with severe sepsis coupled with documented monocyte suppression seemed to benefit from daily subcutaneous IFN-γ (6). Similarly, trauma patients with diminished immune responsiveness, as measured by monocyte major histocompatibility complex class II receptor status, were less likely to develop nosocomial pneumonia when treated with inhaled IFN-γ (27).

IL-12 is a heterodimeric protein composed of 2 subunits, p40 and p35, encoded by unrelated genes (28). Neither subunit has biological activity alone, although a p40 homodimer may act as IL-12 antagonist (28). Furthermore, IL-12 production by monocytes results in a 500-fold excess of p40 relative to the active heterodimer (29). Approximately 20% to 40% of the p40 in the serum of normal and endotoxin-treated mice is in the form of a homodimer (30). In addition, the p40 subunit may lack sensitivity because it forms a common component of other heterodimeric cytokines such as IL-23 (31). As a consequence of this, we chose to measure the p35 subunit as an index of the IL-12 heterodimer activity. Both IL-12 and IL-4 have important roles in $CD4^+$ T-lymphocyte differentiation, being the principal regulators of naive T-cell maturation into T_H1 or T_H2 cells, respectively (32, 33). In this study, the ICU group had a higher IL-12/IL-4 ratio than the bacteremic group. This response seems appropriate, and the net effect should have polarized $CD4^+$ T lymphocyte differentiation into a T_H1 response, characterized by greater IFN-γ production. However, IFN-γ mRNA levels in patients with severe sepsis were lower than those in the control and bacteremic patients. These data are clearly discordant and suggest that, within the pathway of $CD4^+$ T-cell differentiation, the molecular basis of the aberrant $CD4^+$ T_H cell polarization observed in patients with severe sepsis is distal to the antagonistic interaction of IL-12 and IL-4.

One potential limitation of this study lies in the method used for the isolation of PMBCs from whole blood. It is plausible that granulocytes may contaminate the PBMC population during the density gradient purification step because of the similar density of the cell types. Yet because granulocytes produce comparatively very small amounts of IFN-γ, IL-12, and IL-4 (18, 32, 33) and also largely contain much less RNA in comparison to PBMCs, the results presented here remain significant. However, there remains this potential constraint in the interpretation of the TNF-α and IL-10 results.

The data presented in our study use the sensitivity of QRT-PCR as a method for evaluating cytokine gene expression. Prior investigations of cytokine responses to infection relied exclusively on enzyme-linked immunosorbent assay–based protein assays and, with the exception of IL-6, were unable to either detect proinflammatory cytokines in a significant proportion of patients or relate cytokine levels to clinical events (14, 34, 35). Similarly, we were unable to detect serum cytokine protein, other than IL-6, in most patients. IL-6 did distinguish between survivors and nonsurvivors on day 1 of ICU stay and was related to severity of the disease on day 7, which is in keeping with prior studies (34, 35). However, a QRT-PCR–based mRNA assay provides uniquely sensitive *in vivo* information, not relying on *ex vivo* stimulation of cells to produce adequate amounts of material for detection which creates an artifactual setting. Therefore, QRT-PCR allows us to investigate absolute levels of gene expression *in vivo*, which can be used as an index of the adequacy of the cytokine response to infection.

This study demonstrates the use of measuring cytokine mRNA by QRT-PCR in patients with infection. We show a marked association between the pattern of cytokine gene expression and the occurrence of severe sepsis, persistent shock, and survival, with an association between adverse outcome and an anti-inflammatory cytokine response.

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APPENDIX I. **Oligonucleotides for real-time PCR**

mRNA targets	Oligonucleotides (5′→3′)*	Product size, bp	Final concentration, nmol/L†
β-actin	F976: GGATGCAGAAGGAGATCACTG	90	F300; R300
	R1065: CGATCCACACGGAGTACTTG		
	P997: 6Fam-CCCTGGCACCCAGCACAATG-Tamra-p		
IFN-γ	F464: CTAATTATTCGGTAACTGACTTGA	75	F600; R900
	R538: ACAGTTCAGCCATCACTTGGA		
	P491: 6Fam-TCCAACGCAAAGCAATACATGAAC-Tamra-p		
IL-12p35	F212: CTCCTGGACCACCTCAGTTTG	76	F600; R900
	R287: GGTGAAGGCATGGGAACATT		
	P234: 6Fam-CCAGAAACCTCCCCGTGGCCA-Tamra-p		
IL-4	F174: ACTTTGAACAGCCTCACAGAG	74	F300; R900
	R247: TTGGAGGCAGCAAAGATGTC		
	P204: 6Fam-CTGTGCACCGAGTTGACCGTA-Tamra-p		
TNF-α	Supplied as a precustomized mix from Applied Biosystems		F900; R900; P250
IL-10	Supplied as a precustomized mix from Applied Biosystems		F900; R900; P250

*F, R, and P indicate forward and reverse primers and probes, respectively; numbers indicated the sequence position.
†Final concentration of forward and reverse primers and probe.
bp indicates base pair.

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Appendix II. **Oligonucleotides for standard preparation**

mRNA targets	Oligonucleotides (5′→3′)*	Product size, bp	Conditions for "classic" PCR†
IL-10	F296: TTTACCTGGAGAGGTGATG	476	A = 56, Mg = 1.5
	R771: TTGGGCTTCTTTCTAAATCGT		
TNF-α	F83: ACCATGAGCACTGAAAGCAT	406	A = 58, Mg = 1.5
	R488: AGATGAGGTACAGGCCCTCT		
IFN-γ	F154: TTGGGTTCTCTTGGCTGTTA	479	A = 58, Mg = 1.5
	R632: AAATATTGCAGGCAGGACAA		
β-actin	F745: CCCTGGAGAAGAGCTACGA	509	A = 58, Mg = 1.5
	R1253: TAAAGCCATGCCAATCTCAT		
IL-12	F185: AGCCTCCTCCTTGTGGCTA	228	A = 59, Mg = 1.5
	R412: TGTGCTGGTTTTATCTTTTGTG		
IL-4	F27: TAATTGCCTCACATTGTCACT	503	A = 58, Mg = 1.5
	R529: ATTCAGCTCGAACACTTTGAA		

*F and R indicate forward and reverse primers, respectively; numbers indicate the sequence position.
†Conditions, for all targets, were as follows: denaturation at 95°C for 20 s, annealing (temperature as stated [A]) for 20 s and elongation at 72°C for 45 s, for a total of 35 cycles. Magnesium chloride concentration (Mg, mmol/L) was as stated.

Appendix III. **Correlation matrix of white cell subsets to cytokine mRNA in patients with bacteremia and in ICU patients on day 1**

	TNF-α	IFN-γ	IL-12	IL-4	IL-10
WCC	0.04 (0.12)	0.02 (0.27)	0.03 (0.16)	0 (0.91)	0 (0.92)
PMNs	0.02 (0.26)	0 (0.76)	0.05 (0.09)	0 (0.64)	0.005 (0.63)
PBMCs	0 (0.87)	0 (0.76)	0.04 (0.11)	0 (0.64)	0 (0.49)

All correlations were analyzed by linear regression analysis and presented as r^2 values, with significance level in parentheses.
P values are reported as values uncorrected for repeated measures.
PMN indicates polymorphonuclear lymphocytes; WCC, white cell count.

Appendix IV. **Correlation matrix of white cell subsets to cytokine mRNA on day 7 for the ICU group**

	TNF-α	IFN-γ	IL-12	IL-4	IL-10
WCC	0.05 (0.1)	0.08 (0.06)	0 (0.92)	0 (0.75)	0 (0.67)
PMNs	0.06 (0.1)	0.08 (0.06)	0 (0.92)	0.005 (0.6)	0.01 (0.48)
PBMCs	0.002 (0.74)	0.02 (0.36)	0.003 (0.69)	0.02 (0.3)	0.03 (0.28)

All correlations were analyzed by linear regression analysis and presented as r^2 values, with significance level in parentheses.
P values are reported as values uncorrected for repeated measures.
PMN indicates polymorphonuclear lymphocytes; WCC, white cell count.

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Research

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Septic shock is correlated with asymmetrical dimethyl arginine levels, which may be influenced by a polymorphism in the dimethylarginine dimethylaminohydrolase II gene: a prospective observational study

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Abstract

Introduction Asymmetrical dimethyl arginine (ADMA) is an endogenous non-selective inhibitor of nitric oxide synthase that may influence the severity of organ failure and the occurrence of shock secondary to an infectious insult. Levels may be genetically determined by a promoter polymorphism in a regulatory gene encoding dimethylarginine dimethylaminohydrolase II (DDAH II), which functions by metabolising ADMA to citrulline. The aim of this study was to examine the association between ADMA levels and the severity of organ failure and shock in severe sepsis and also to assess the influence of a promoter polymorphism in *DDAH II* on ADMA levels.

Methods A prospective observational study was designed, and 47 intensive care unit (ICU) patients with severe sepsis and 10 healthy controls were enrolled. Serum ADMA and IL-6 were assayed on admission to the ICU and seven days later. Allelic variation for a polymorphism at position -449 in the *DDAH II* gene was assessed in each patient. Clinical and demographic details were also collected.

Results On day 1 more ADMA was detectable in the ICU group than in the control group ($p = 0.005$). Levels subsequently increased during the first week in ICU ($p = 0.001$). ADMA levels were associated with vasopressor requirements on day one ($p = 0.001$). ADMA levels and Sequential Organ Failure Assessment scores were directly associated on day one ($p = 0.0001$) and day seven ($p = 0.002$). The degree of acidaemia and lactaemia was directly correlated with ADMA levels at both time points ($p < 0.01$). On day seven, IL-6 was directly correlated with ADMA levels ($p = 0.006$). The variant allele with G at position -449 in the *DDAH II* gene was associated with increased ADMA concentrations at both time points ($p < 0.05$).

Conclusion Severity of organ failure, inflammation and presence of early shock in severe sepsis are associated with increased ADMA levels. ADMA concentrations may be influenced by a polymorphism in the *DDAH II* gene.

Introduction

Overwhelming infection with resultant multiple organ failure, which has been termed the 'sepsis syndrome' [1], is a devastating illness, and a common intensive care unit (ICU) admission diagnosis, with an incidence of 3 per 1,000 population per annum [2]. The sepsis syndrome has been characterised as a dysregulation of inflammation in response to infection, with life-threatening organ failure attributable to a combination of excessive inflammation, disseminated coagulopathy and disruption of the integrity of microvascular endothelium [3].

ADMA = asymmetrical dimethyl arginine; DDAH = dimethylarginine dimethylaminohydrolase; ELISA = enzyme-linked immunosorbent assay; eNOS = endothelial NO synthase; iNOS = inducible NO synthase; ICU = intensive care unit; IL = interleukin; NO = nitric oxide; NOS = nitric oxide synthase; SOFA = Sequential Organ Failure Assessment.

Endothelium-derived nitric oxide (NO) is a potent vasodilator that antagonises the effects of endogenous vasopressors [4]. NO is produced from L-arginine by an enzyme, nitric oxide synthase (NOS), which exists in constitutive, inducible, endothelial and neuronal isoforms. The endothelial isoform (eNOS) regulates vascular tone and interactions between leukocytes and endothelium [5]. Consequently, NO has been implicated in the pathogenesis of the hypotension and organ failure attributable to severe sepsis [6]. However, although non-selective pharmacological inhibition of NOS briefly attenuates the haemodynamic anomalies seen in these patients with severe sepsis, the overall effect of such inhibition is to increase mortality [7].

This conundrum may be explained in part either by selective inhibition of the various isoforms of NOS or by an ancillary non-vascular function of NOS. Specifically, inhibition of the constitutively expressed isoform of NOS, which is essential to maintain organ perfusion, may be detrimental [8]. However, and of considerably greater importance in the context of sepsis, NO has an ancillary yet critical protective function, possessing potent antimicrobial properties, antagonism of which may account for the excess mortality observed with NOS inhibition in patients with sepsis [9].

Asymmetrical dimethyl arginine (ADMA) is a naturally occurring non-selective inhibitor of NOS, derived from protein catabolism, and is metabolised to citrulline by dimethylarginine dimethylaminohydrolase (DDAH) [10]. The co-localisation of DDAH and NOS at several sites supports the hypothesis that DDAH may regulate NOS activity by controlling the metabolism of ADMA [10]. DDAH exists as two distinct isoforms, with DDAH I present in tissues expressing neuronal *NOS*, whereas *DDAH II* has an expression pattern similar to that of eNOS [11], thus making DDAH II characteristic of vascular tissue such as the heart and endothelium. Variation in *DDAH II* expression or activity might therefore be an important mechanism in the haemodynamic alterations and end-organ damage observed in sepsis. Notably, DDAH displays decreased activity when operating in an inflammatory milieu [12]. Depletion of NO by ADMA has biological significance, because elevated ADMA levels are seen in patients with vascular disease, hepatic failure and renal failure, and are linked with greater severity of organ failure in ICU patients with sepsis [5,13]. Furthermore, it has recently been postulated that the beneficial effects of the administration of exogenous insulin may be associated with fluctuations in ADMA levels in patients with sepsis [13]. However, variation in ADMA levels may also have a genetic basis. Gene polymorphism, observed in the promoter region of the *DDAH II* gene, may have functional significance [14] but has not previously been studied in a human population with sepsis. However, an association between gene polymorphism in the promoter region of the *DDAH II* gene and systemic arterial vasodilation after cardiac surgery with cardiopulmonary bypass suggests a link between pathological vasodilation, such as that occuring with severe sepsis, and ADMA metabolism [15].

We undertook a study to assess the relationship between ADMA levels and organ failure in ICU patients with severe sepsis and also to assess the possible functionality of a polymorphism in the *DDAH II* promoter, designated *DDAH II* -449 (single-nucleotide polymorphism (SNP) ID rs805305).

Materials and methods

This study was conducted in the ICU of St James's Hospital, Dublin, Ireland, and was approved by the local research ethics committee. Informed written consent was obtained from each patient or a first-degree relative. A total of 47 consecutive patients with severe sepsis or septic shock, as defined by the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference [1] were enrolled. Ten healthy staff members served as a control group.

Severity of illness was characterised with the Sequential Organ Failure Assessment (SOFA) scoring system [16] and the Simplified Acute Physiology Score (SAPS2) [17] on admission to ICU, and with the SOFA score again on day seven. Individual clinical and laboratory variables relating to inflammation were collected on days one and seven of ICU stay. The recorded variables represented the most significant derangements from normal values recorded over each 24-hour period. The requirement for vasoactive or vasopressor medications to maintain a mean arterial pressure greater than 60 mmHg was recorded. These medications consisted of either adrenaline or noradrenaline infusions. Death in ICU or survival to ICU discharge was recorded.

Blood sampling was performed within the first 24 hours of ICU admission and again seven days later through an indwelling central venous line. Serum was obtained from whole blood clotted for 30 minutes at room temperature and spun at 2,500 rev./minute for 10 minutes.

ADMA was measured with a microtitre plate assay (DLD Diagnostika Ltd, Hamburg, Germany) as described previously [18].

Serum IL-6 concentrations were measured by ELISA (R&D Systems, Minneapolis, MN, USA) in accordance with the manufacturer's instructions. The lower limit of detection for IL-6 was 9.4 pg/ml. All samples were tested in duplicate.

Genomic DNA was extracted from whole blood with a commercially available DNA isolation kit (QIAmp DNA blood Midi kit, Qiagen GmBH, Crawley, West Sussex, UK). Allelic variation for the polymorphism was assayed using Amplifluor technology by Kbiosciences (Hoddesdon, Herts., UK). Primer sequences are listed in Table 1.

Table 1

Locus		Primer
DDAHII-449	Allele 1	GAAGGTGACCAAGTTCATGCTGACTGGAAGTCCAGCCCGG
	Allele 2	GAAGGTCGGAGTCAACGGATTGACTGGAAGTCCAGCCCGC
	Common	CCAGCTTTCTCCTTCTGTCCCATAA

Table 2

Demographics and asymmetrical dimethyl arginine (ADMA) levels by group

Parameter	Survivors	Non-survivors	*p*
Total patients	33 (70)	14 (30)	
Male sex	17 (52)	9 (64)	ns
SOFA score, day 1	7 (4–10)	9 (8.75–12.5)	0.02
SOFA score, day 7	4 (2.75–7.25)	10.5 (8.5–11.75)	0.006
SAPS2 score	39 (30.5–51.5)	47.5 (39.5–60.75)	ns
Lactate, day 1	2.1 (1.5–5)	3.6 (2.25–5.75)	ns
Lactate, day 7	1.5 (1–1.9)	2 (1.1–4)	ns
Vasoactive agents, day 1	18 (55)	13 (93)	0.01
Vasoactive agents, day 7	5 (15)	5 (63)	0.005
ADMA, day 1	0.88 (0.52–1.09)	0.91 (0.64–1.23)	ns
ADMA, day 7	1.05 (0.66–1.21)	1.24 (0.77–1.53)	ns
pH, day 1	7.31 (7.26–7.38)	7.33 (7.27–7.37)	ns
pH, day 7	7.44 (7.40–7.45)	7.32 (7.24–7.40)	0.002
WCC, day 1	18 (10.7–23)	9.1 (2.3–17.8)	ns
WCC, day 7	11.6 (8.6–17.4)	13.9 (8.5–19.3)	ns
Base excess, day 1	-2.9 (-6.45 to 1.75)	-4.1 (-9.3 to 1.75)	ns
Base excess, day 7	2.9 (1.1–5.6)	0.1 (-3 to 1.33)	0.009
IL-6, day 1	277 (107–499)	782 (566–1,060)	0.001
IL-6, day 7	22 (0–651)	130 (68–425)	ns

All values are shown either as absolute counts with percentages in parenthesis or as medians with interquartile ranges in parenthesis. ADMA was measured in μmol/l, lactate in mmol/l, and IL-6 in pg/ml. SOFA, Sequential Organ Failure Assessment score; SAPS, simplified acute physiology score; ADMA, asymmetrical dimethyl arginine; WCC, white cell count; ns, not significant.

Statistical analysis was performed with the JMP software package (SAS, Cary, NC, USA). Between-group comparisons for continuous variables were analysed by Wilcoxon rank sum test, Wilcoxon sign rank test and Kruskal-Wallis test where appropriate. Spearman's rank correlation coefficient was used to analyse the relationship between continuous variables. For all comparisons, $p < 0.05$ was considered significant.

Results

Consent was gained for 47 ICU patients and from 10 healthy controls; they were recruited into the study. Blood samples were available for analysis from 40 patients on day 1 and from 35 patients on day 7; 28 patients had blood samples available for analysis at both time points. Fourteen (30%) patients died before discharge from ICU. Demographic data, clinical details and levels of inflammatory markers for patients are detailed in Tables 2 to 4.

Day one comparisons

On day one, 31 patients (66%) required infusion of a vasoactive compound to maintain adequate arterial pressure. ADMA levels ($p = 0.001$), lactate levels ($p = 0.018$) and organ failure scores ($p < 0.003$) were higher in this group requiring vasoactive infusions (Table 3). Patients in this group on day one were also more likely to be non-survivors ($p = 0.01$; Table 3).

Plasma lactate levels were directly correlated with ADMA levels on day 1 ($r^2 = 0.28$, $n = 40$, $p = 0.0003$). In addition, SOFA

Table 3

Requirement for vasoactive infusions on day 1

Parameter	Vasoactive infusions	No vasoactive infusions	p
Total patients	31 (66)	16 (34)	
Death	13 (42)	1 (6)	0.01
IL-6	354 (189–768)	293 (87–657)	ns
pH	7.31 (7.26–7.35)	7.32 (7.29–7.40)	ns
Lactate	3.5 (1.9–6.02)	1.8 (1.05–3.75)	0.018
Base excess	-3.5 (-9 to 3.4)	-2 (-4.95 to 0.55)	ns
SOFA	9 (8–12)	4 (3.25–4.75)	<0.0001
SAPS2	47 (38–63)	33.5 (21–41.75)	0.003
ADMA	0.96 (0.82–1.29)	0.54 (0.48–0.78)	0.001
WCC	14 (8–22)	17 (8–24)	ns
MAP	65 (60–70)	79 (66–80)	0.001
Heart rate	110 (90–120)	103 (96–118)	ns
CVP	13 (10–16)	11 (7–12)	ns
Noradrenaline	13 (5–26)	-	

All values are shown either as absolute counts with percentages in parenthesis or as medians with interquartile ranges in parenthesis. IL-6 was measured in pg/ml, asymmetrical dimethyl arginine (ADMA) in μmol/l, lactate in mmol/l, and mean arterial pressure (MAP) and central venous pressure (CVP) in mmHg. Noradrenaline dosage was measured in μg/minute. SOFA, Sequential Organ Failure Assessment score; SAPS, simplified acute physiology score; WCC, white cell count; ns, not significant.

score and ADMA levels were directly correlated on day 1 ($r^2 = 0.31$, $n = 40$, $p < 0.0001$).

To elucidate whether the relationship between ADMA levels and SOFA score was entirely attributable to cardiovascular failure, a non-cardiac organ failure score was obtained by excluding the cardiovascular component from the total SOFA score. There was a positive correlation between this score and ADMA levels on day 1 ($r^2 = 0.23$, $n = 40$, $p = 0.002$).

ADMA levels on day 1 were not related to survival, nor were the highest producers of ADMA (highest quartile) more likely to have a higher mortality. However, SOFA scores and IL-6 levels on day one did distinguish between survivors and non-survivors on day 1 ($p = 0.02$ and $p = 0.001$, respectively) (Table 2).

Day seven comparisons

On day seven, 10 patients (24%) required infusion with vasoactive medication to maintain a normal blood pressure. Although there was a trend towards increasing ADMA levels in those patients requiring vasoactive infusions to maintain blood pressure, this did not reach significance ($p = 0.07$; Table 4).

Plasma lactate levels were directly correlated with ADMA levels on day 7 ($r^2 = 0.18$, $n = 31$, $p = 0.01$). In addition, SOFA score and ADMA levels were directly correlated on day 7 ($r^2 = 0.23$, $n = 35$, $p = 0.002$). The non-cardiac organ failure score was calculated as above from the day 7 SOFA score. This score was positively correlated with ADMA levels on day 7 ($r^2 = 0.22$, $n = 35$, $p = 0.005$).

Table 4

Use of vasoactive infusions on day 7

Parameter	Vasoactive infusions	No vasoactive infusions	p
Total patients	10 (24)	31 (76)	
Death	5 (50)	3 (10)	0.005
IL-6	299 (20–784)	23 (0–117)	0.037
pH	7.36 (7.23–7.41)	7.44 (7.40–7.45)	0.0006
Lactate	2.35 (1.48–3.73)	1.2 (1–1.8)	0.006
Base excess	0.05 (4.7–3.8)	2.9 (1.1–4.8)	0.04
SOFA	11 (10–15)	4 (2.3–5.8)	<0.0001
ADMA	1.21 (0.88–1.57)	1 (0.66–1.18)	ns
WCC	18.2 (13.3–22.2)	10 (8.2–13.8)	0.01
MAP	70 (69–76)	82 (80–90)	0.01
Heart rate	100 (85–111)	78 (70–90)	0.04
CVP	11 (10–12)	10 (9–14)	ns
Noradrenaline	12 (4–21)	-	

All values are shown either as absolute counts with percentages in parenthesis or as medians with interquartile ranges in parenthesis. IL-6 was measured in pg/ml, asymmetrical dimethyl arginine (ADMA) in μmol/l, lactate in mmol/l, and mean arterial pressure (MAP) and central venous pressure (CVP) in mmHg. Noradrenaline dosage was measured in μg/minute. SOFA, Sequential Organ Failure Assessment score; WCC, white cell count; ns, not significant.

Table 5

Asymmetrical dimethyl arginine (ADMA) levels by group

Parameter	Control	Day 1 ICU[a]	Day 7 ICU[b]
ADMA	0.63 (0.57–0.71)	0.89 (0.57–1.09)	1.05 (0.71–1.32)

All values are in μmol/l and are presented as medians with interquartile ranges in parenthesis. ICU, intensive care unit. [a]Comparison between day 1 ICU and control group by Wilcoxon rank sum test; $p = 0.005$. [b]Comparison between day 1 ICU and day 7 ICU by Wilcoxon signed rank test; $p = 0.001$.

ADMA levels on day 7 were not related to survival, nor were the highest producers of ADMA (highest quartile) more likely to have a higher mortality. However, increased SOFA scores, acidosis and requirement for infusion of vasoactive medications on day 7 were associated with increased risk of death (Table 2).

ADMA levels by group

On the first day of critical illness, the ICU group had greater ADMA levels than the control group ($p = 0.005$). ADMA levels

Table 6

Correlation matrix of asymmetrical dimethyl arginine (ADMA) and inflammatory markers

Parameter	ADMA day 1 data	ADMA day 7 data
pH	0.31 (0.0002)	0.32 (0.001)
Base excess	0.13 (0.02)	0.24 (0.005)
Lactate	0.29 (0.0003)	0.20 (0.01)
WCC	ns	ns
IL-6	ns	0.25 (0.006)

Values are r^2 with p values in parenthesis. WCC, white cell count; ns, not significant.

Table 7

Multivariate linear regression between SOFA scores and ADMA and IL-6 levels

Parameter	*F* ratio	*p*
Day 1 ($n = 36$, $r^2 = 0.35$)		
ADMA	11	0.002
IL-6	7.8	0.009
Day 7 ($n = 30$, $r^2 = 0.32$)		
ADMA	12.02	0.002
IL-6	0.88	ns

SOFA, Sequential Organ Failure Assessment score; ADMA, asymmetrical dimethyl arginine; ns, not significant.

subsequently rose over the first week in the ICU group ($p = 0.001$; Table 5).

Correlation between ADMA levels and inflammatory markers

Various inflammatory markers were correlated with ADMA levels on univariate analysis on both day 1 and day 7 (Table 6). Whereas pH, base excess and lactate levels were correlated with ADMA on both day 1 and day 7, IL-6 levels were correlated with ADMA only on day 7, and the white cell count was not correlated with ADMA at either time point (Table 6).

Correlation between severity of organ failure and ADMA and IL-6 levels

Multivariate analysis of the relationship between the SOFA scores and the biological markers ADMA and IL-6 revealed that on day 1 both ADMA ($p = 0.002$) and IL-6 ($p = 0.009$) were independently related to SOFA scores, whereas on day 7 only ADMA ($p = 0.002$) was independently related to the SOFA score (Table 7).

Allelic variations

The distribution of *DDAH II* alleles conformed to a Hardy-Weinberg equilibrium. There was no association between any clinical outcome measure and carriage of specific *DDAH II* alleles. Twenty-four patients (45%) were GG homozygotes, 5 (11%) were CC homozygotes and 19 (43%) were heterozygotes at position -449 in the *DDAH II* promoter. There was a trend towards increasing amounts of ADMA between different *DDAH II* genotypes. ADMA was most abundant in the GG homozygotes, least abundant in the CC homozygotes and detectable at intermediate levels in the heterozygotes. This trend was present at both time points, although it failed to reach significance on either day 1 ($p = 0.069$) or on day 7 ($p = 0.32$). However, carriage of the G allele at position -449 was associated with increased ADMA production on both day 1 ($p = 0.03$) and day 7 ($p = 0.042$) (Table 8).

Table 8

Variation in asymmetrical dimethyl arginine (ADMA) levels with carriage of specific alleles at DDAH II -449

Day	ADMA (μmol/l)		*p*
	GC/GG genotype	CC genotype	
1	0.91 (0.63–1.16)	0.51 (0.45–0.70)	0.03
7	1.06 (0.77–1.35)	0.835 (0.67–1.03)	0.04

Values are medians with interquartile ranges in parenthesis. Patients carrying variant allele with G at position -449 have either a GG or a GC genotype. DDAH, dimethylarginine dimethylaminohydrolase.

Discussion

There are limited data on the role of ADMA and DDAH II in systemic inflammation, with two studies of critically ill patients observing a relationship between the highest producers of ADMA and fatal outcome [5,13]. Although our study may not have been adequately powered to detect outcome variations, we have demonstrated both an increase in ADMA levels in critically ill patients in comparison with healthy controls and described an association between increasing ADMA levels, the occurrence of septic shock and greater severity of organ failure.

Given the ubiquitous involvement of NO in vascular regulation and leukocyte function, the consequences of excess ADMA in inflammatory and septic states are likely to be manifold. Raised ADMA levels may lead to pathogenic changes in the microvasculature by inhibiting constitutively expressed NOS [8]. The consequent loss of basal NO production may lead to impaired blood flow with platelet aggregation, causing endothelial damage, interstitial oedema and resultant organ failure [19].

However, ADMA mediated inhibition of inducible NOS (iNOS) in patients with sepsis may interfere with macrophage bactericidal properties, because NO is an essential component in the phagocytic response to bacterial infection. Interferon-γ, released in response to an infective insult, acts on macrophages to increase the expression of iNOS [9]. This activates the cells to a heightened microbicidal state, mediated by NO and adducts of the nitrogenous products of nitric oxide synthases. As a consequence mice with a non-functional *iNOS* gene are susceptible to infection [20]. Furthermore, in clinical

trials of NOS inhibition in patients with sepsis, although NOS inhibition ameliorates pathogenic vasodilation and lessens vasopressor requirement, the overall effect is to compromise survival [7]. This suggests, in the context of severe sepsis, that NO-linked immune mechanisms are of greater importance than NO-meditated vascular regulation.

We observed that elevated ADMA levels are correlated with vasopressor support in early septic shock. Although this may seem counterintuitive because previous evidence implicated NO in the pathogenesis of the hypotension observed in septic shock [21], it is plausible that inappropriately increased ADMA levels may impair macrophage function by means of NOS inhibition. The associated inflammatory response to an unresolved infection may be partly responsible for the observed hypotension and organ failure operating through an alternative mechanism. This persistent inflammatory response is reflected in the linkages between IL-6, ADMA and the severity of organ failure (Tables 6 and 7). The association with IL-6 is noteworthy because this is a well-recognised marker of generalised inflammation, consistently elevated in patients with sepsis [22].

About 90% of ADMA is metabolised by the enzyme DDAH [10]. It is possible that variation in ADMA levels in patients with sepsis is reactive and represents an epiphenomenon. However, we observed that carriage of a G at position -449 in the promoter region of the *DDAH II* gene is associated with increased ADMA levels, which suggests that the *DDAH II* gene with a G at this position is less active than that with a C. The more active isoform results in lower ADMA levels, less iNOS inhibition and consequently an appropriate bactericidal phagocytic response. It is noteworthy that *DDAH II* maps to 6p21.3, a region of DNA that is particularly rich in genes involved in immune and inflammatory responses. It has been hypothesised that this location and wide expression in immune cells make it a candidate as a disease susceptibility gene in sepsis [10].

We have previously described an association between the presence of a G at position -449 in the *DDAH II* gene and the requirement for vasopressors after cardiopulmonary bypass during cardiac surgery [15]. Although this is the opposite of what we observed in septic patients, it is noteworthy that the two insults are also quite different. The cardiopulmonary bypass circuit invokes a sterile inflammatory response, whereas the ICU patients with sepsis received an infective inflammatory insult. Consequently, the role of ADMA in manipulating NO levels may be context sensitive. NO may have pivotal beneficial bactericidal properties necessary for the resolution of a septic insult while contributing to an undesirable vasodilatory state in the setting of a sterile inflammatory insult.

This potential genetic component to the fluctuations observed in ADMA levels secondary to a septic insult may help to explain some of the residual variability observed in a previous study attempting to link exogenous insulin administration to ADMA levels [13]. Thus, interindividual variability in ADMA production is likely to be multifactorial, with contributions from genetic and environmental factors.

Conclusion

We have confirmed the association between ADMA levels and the extent of multiple organ failure in sepsis. We have also demonstrated that ADMA levels are upregulated in response to an infective insult and are also associated with hypotension in this setting. We hypothesise that this may be due to ineffective bactericidal activity of macrophages and persistent inflammation. Finally, we suggest that ADMA levels may be regulated via a genetic component. We propose that a polymorphism at position -449 in the *DDAH II* may be functional and has the potential to be used as a marker for the susceptibility to and severity of an inflammatory response secondary to an infective insult. A larger study will be required to confirm these findings.

Key messages

- ADMA, an endogenous non-selective inhibitor of NOS, may have a key role in vascular regulation.
- Compromised NO production may influence morbidity by disrupting microcirculatory blood flow and also could potentially compromise key bactericidal functions in the host.
- Increased ADMA levels are associated with multiple organ failure and shock in the setting of a septic insult.
- ADMA may be regulated by means of host genetic mechanisms, which influence the efficiency of the enzymatic breakdown of ADMA by *DDAH II*.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MO'D participated in the design of the study, patient recruitment, data and sample collection, ELISA and DNA analysis, statistical analysis, and drafting of the manuscript. FD and VC participated in the ADMA analysis. DK participated in the design of the study and drafting of the manuscript. RM participated in the design of the study, genotype analysis, statistical analysis and drafting of the manuscript. TR participated in the design of the study, patient recruitment, statistical analysis and drafting of the manuscript. All authors read and approved the final manuscript.

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Abstract *Objective:* The development and progression of severe sepsis is related to a deficiency in pro-inflammatory cytokine production, characterised by lesser IFNγ levels, which are not explained by variations in levels of the main putative regulator of IFNγ, namely IL-12. As alternative regulators of IFNγ may be of greater importance in human sepsis, we investigated the hypothesis that the development of severe sepsis is related to variations in IL-18, IL-23 and IL-27 gene expression. *Design and setting:* A prospective observational trial in a mixed intensive care unit (ICU) and hospital wards in a university teaching hospital. *Patients and participants:* Sixty-two ICU patients with severe sepsis, 13 bacteraemic patients with no acute critical illness, and 10 healthy controls. *Measurements and results:* All subjects were assayed for IL-18, IL-23 and IL-27 mRNA levels in peripheral blood. IL-27 mRNA levels distinguished between the three groups, with levels highest in the ICU group, intermediate in the bacteraemic group and lowest in the control group. IL-23 distinguished between the groups, with levels lowest in the ICU group. In late sepsis IL-23 and TNFα mRNA levels were directly related. IL-18 mRNA levels did not distinguish between the patient groups. *Conclusions:* We conclude that the deficient pro-inflammatory response in patients with sepsis is expansive and includes deficient IL-23 and excessive IL-27 gene expression. This provides further evidence that upregulation of a cytokine-based immune response is beneficial in sepsis.

Keywords Sepsis syndrome · Cytokines · Reverse transcriptase polymerase chain reaction · Helper T-cells

Introduction

The human response to infection ranges from a mild subclinical illness to life-threatening septic shock. In humans the development of severe sepsis has been linked with a profound immune paresis [1, 2]. We have previously demonstrated that one aspect of the molecular basis of this immune paresis is an increase in anti-inflammatory cytokine gene expression, principally interleukin (IL) 10, and a decrease in interferon (IFN) γ and tumour necrosis factor (TNF) α gene expression [3]. Additionally, we have described an association between mortality

and deficient IFNγ messenger RNA (mRNA) levels in sepsis.

IFNγ is produced by natural killer cells, monoyctes and CD4^{+} T helper cell type 1 (Th1) cells. In these cells the pre-eminent cytokine influencing the production of IFNγ, is considered to be IL-12 [4]. Interestingly, in our previous study variability in IL-12 mRNA levels accounted for only a minority of the variability in IFNγ gene expression, and we failed to detect a relationship between IL-12 mRNA levels and outcome [3]. However, it has recently been appreciated that IFNγ production in response to infection is regulated by a number of additional cytokines. These include IL-18, originally termed IFNγ-inducing factor, and two homologs of IL-12, namely IL-23 and IL-27 [5, 6]. In addition, the latter two pleiotropic cytokines have recently been reported as being antagonistic regulators of CD4^{+} T cells that have a Th17 phenotype and may therefore mediate the immune response to infection via this novel pathway [7].

We hypothesised that variability in the cytokine regulators of IFNγ production, other than IL-12, may prove important in the human response to infection. We performed a study to determine whether the nature of response to an infectious insult and patient outcome from severe sepsis are linked with distinct patterns of IL-18, IL-23 and IL-27 gene expression. In this study we used a methodology incorporating quantitative real-time polymerase chain reaction (QRT-PCR) to provide an absolute quantification of specific cytokine messenger RNA (mRNA) copy numbers. The suitability of QRT-PCR as a method of quantification of in vivo cytokine gene expression in human sepsis is becoming increasing recognised as an appropriate and acceptable assay [3, 8]. This technique is extremely sensitive and when used in humans with infection can detect a link between distinct patterns of cytokine mRNA and outcome in patients without recourse to exogenous stimulation with adjuvant agents such as lipopolysaccharide [3]. Whilst the lack of sensitivity associated with protein assays in this setting is well known [3, 9, 10] a recent report confirms the suitability of mRNA quantification as a surrogate of protein expression [11].

Materials and methods

Patients

This study was conducted in St James's Hospital, Dublin, Ireland, and was approved by the institutional ethics committee. Informed written consent was obtained from each patient or a relative. Exclusion criteria included: (a) pre-existing overt organ failure, (b) infection with the human immunodeficiency virus, (c) patients neutropenic as a result of chemotherapy, (d) patients receiving long-term treatment with corticosteroids, (e) trauma and burns patients, and (f) non-Irish white ethnic background. Three patient groups were recruited:

Group 1: ICU group

A total of 62 consecutive intensive care unit (ICU) patients presenting with severe sepsis or septic shock [12] as a primary admission diagnosis were enrolled over 12 months. All ICU patients received similar standardised care. Severity of illness was characterised on admission to the ICU using the Sequential Organ Failure Assessment (SOFA) scoring system [13] and the Simplified Acute Physiology Score 2 (SAPS II) [14] and again on day 7 of ICU stay using the SOFA score. Individual clinical and laboratory variables were collected on day 1 and day 7 of ICU stay. The recorded variables represented the most significant derangements from normal values recorded over each 24-h period. ICU death or survival to ICU discharge was recorded. Shock was defined as the requirement for vasopressors to maintain a mean arterial pressure greater than 60 mmHg. Vasopressors referred to adrenaline and/or noradrenaline. The demographics of this patient group have been previously discussed [3] and are presented in Tables 1 and 2. ICU patients were culture negative for their admitting infective insult in 39% of cases ($n=24$). Where an organism was isolated, Gram-positive organisms were the most common (66%), followed by Gram-negative organisms (43%) and fungal pathogens (7%). Isolation of an individual pathogen was not associated with any particular profile

Table 1 Demographic and clinical characteristics of septic shock and severe sepsis patients on day 1 and bacteraemic patients (*SOFA*, Sequential Organ Failure Assessment [13]; *SAPS*, Simplified Acute Physiology Score [14]; *IQR*, interquartile range)

	Septic shock ($n=42$)	Severe sepsis ($n=20$)	Bacteraemia ($n=10$)	p
Males	26 (61%)	10 (50%)	4 (40%)	0.37
Age, median (years; IQR)	70 (56–77)	62 (43–72)	82 (62–85)	0.017
SOFA score, median (IQR)	9 (8–12)	4 (3–5)	0.5 (0–1)	<0.0001
SAPS II, median (IQR)	47 (39–59)	35 (28–42)	–	0.0008
Duration of ventilation, median (days)	9 (3–21)	2 (0–17)	–	0.049
Duration of ICU stay, median (days)	11 (4–25)	5 (2–22)	–	0.08
Site of infection				
Respiratory	21 (50%)	11 (55%)	4 (40%)	0.74
Abdominal	14 (33%)	7 (35%)	3 (30%)	0.67
Other	8 (19%)	2 (10%)	3 (30%)	0.48

Table 2 Survivors vs. non-survivors from the ICU group (*SOFA*, Sequential Organ Failure Assessment [13]; *SAPS*, Simplified Acute Physiology Score [14]; *IQR*, interquartile range)

	Survivors ($n = 45$)	Non-survivors ($n = 17$)	p
Males	24 (53%)	10 (59%)	0.78
Age, median (years; IQR)	62 (19–81)	67 (39–86)	0.55
SOFA day 1, median (IQR)	7 (4–10)	9 (9–13)	0.003
SAPS II, median (IQR)	40 (33–51)	49 (41–60)	0.02
Ventilated day 1	36 (80%)	17 (100%)	0.05
Shock day1	26 (58%)	16 (94%)	0.006
ICU stay, median (days; IQR)	12 (3.5–27)	5 (3–10.5)	0.03
Site of infection			
Respiratory	22 (49%)	10 (59%)	0.57
Abdominal	15 (33%)	5 (29%)	0.88
Others	8 (18%)	2 (12%)	0.53

of cytokine mRNA levels nor was a particular class of pathogen associated with any profile of cytokine mRNA levels.

Group 2: bacteraemia group

Ten consecutive patients from hospital wards with a documented Gram-negative bacteraemia, confirmed on blood culture, were identified by the microbiology department and were enrolled if no organ failure or impending septic crisis were identified. The specific presence of a Gram-negative isolate was deemed the most appropriate method of eliminating potential contaminated blood culture samples.

Group 3: control group

Thirteen healthy staff members served as a control group.

Blood sampling

Blood sampling was carried out within the first 24 h of ICU admission and again 7 days later. In the bacteraemia group blood sampling was carried out within 24 h of the positive blood culture being reported. Blood samples were collected from healthy controls at one time point. The buffy coat layer was isolated from peripheral blood samples and RNA was isolated from this layer and reverse transcribed as previously described [3]. Blood samples were available for PCR analysis from 52 of the patients with severe sepsis or septic shock on the first day of critical illness and from 49 of these patients on the seventh day of critical illness. A total of 39 patients had blood samples available for PCR analysis at both time points.

Primers and probes

All primer and probes used in this study were synthesised at Applied Biosystems (Foster City, CA, USA). IL-27 primers and probes were obtained as a pre-customised mix (assay ID for IL-27 is Hs00377366_m1). β-Actin, IL-18 and IL-23p19 primers and probes were designed and customised (sequences listed in Appendix 1, Electronic Supplementary Material, ESM) as per Stordeur et al. [15].

Quantitative real-time PCR

The QRT-PCR reactions were carried out in an ABI Prism 7000 (Applied Biosytems). All reactions were performed either in triplicate or in duplicate. Thermocycling was carried out in a 20 μl final volume as previously described [3].

Standard curves and expression of the results

Preparation of the DNA standards and standard curves have been previously described [3]. All standard curves showed correlation coefficients higher than 0.99. The efficiency of the standard curves for all target cDNA was greater than 96%. Results were expressed in absolute copy numbers after normalisation against β-actin mRNA (copy numbers of cytokine mRNA per 10 million β-actin mRNA copy numbers).

Statistical analysis

The Wilcoxon rank sum test was used to analyse the differences between groups for continuous variables. Categorical variables were analysed by χ^2 test and Fisher's exact test as appropriate. The correlation between continuous variables was analysed using Spearman's rank correlation coefficient. Data analysis was performed using the JMP statistical software package (SAS, Cary, NC, USA).

Results

Day 1 comparisons between the three groups

There was a difference in IL-23 mRNA levels when the three groups were analysed together ($p = 0.03$), with the severe sepsis/septic shock group demonstrating the lowest

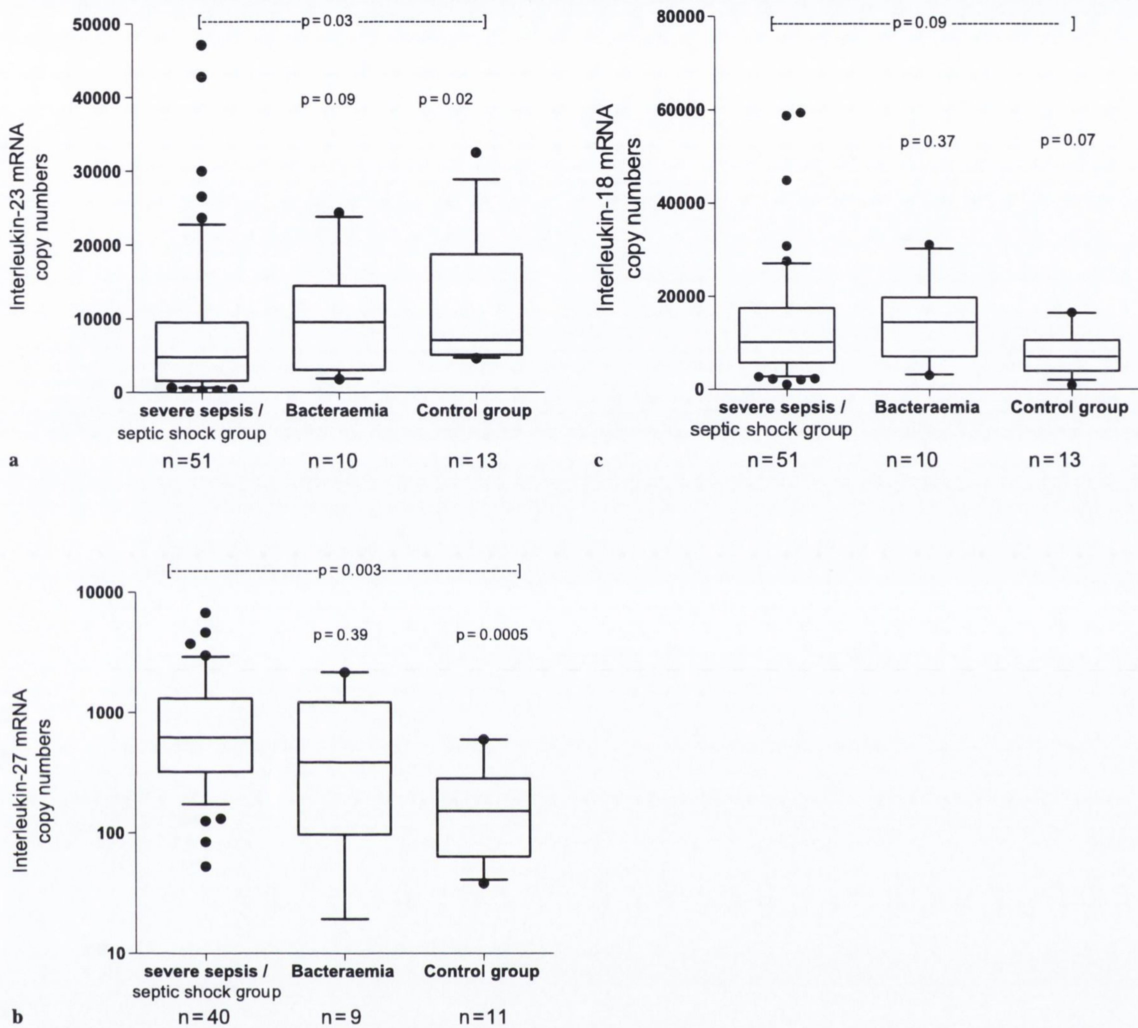

Fig. 1 Cytokine mRNA levels across the ICU group with severe sepsis or septic shock, the non-critically ill bacteraemia group and the control group. IL-23 (**a**), IL-27 (**b**) and IL-18 (**c**) expressed as copy numbers of cytokine mRNA per 10 million β-actin mRNA copy numbers. Values, represented as box plots, are median, quartiles 25–75% (*box*), and centiles 10–90% (*whiskers*). Individual *p*-values are for comparison with the ICU group. *The p value at the top of the figure* represents a threegroup comparison (Kruskal–Wallis test); *ns*, non-significant

IL-23 mRNA levels (Fig. 1a). There was a difference in IL-27 mRNA levels between the three groups ($p = 0.003$), with the severe sepsis/septic shock group having the greatest levels followed by the bacteraemic group with the control group having the least amount of detectable IL-27 mRNA (Fig. 1b). IL-18 mRNA levels did not distinguish between the three groups (Fig. 1c). Quantitative data for the differences in mRNA levels between the groups are presented in Appendix 2 (ESM).

Severity of illness and mRNA levels in the severe sepsis/septic shock group

In the severe sepsis/septic shock group, no association was detected between cytokine mRNA levels and the presence or absence of shock or the severity of disease, as assessed by the SOFA score, on day 1. In the severe sepsis/septic shock group on day 7 there was an inverse correlation between IL-23 mRNA levels and SOFA score

Table 3 Cytokine mRNA levels and outcome in the severe sepsis/septic shock group. Results are expressed as mRNA copy numbers per 10 million β actin mRNA copies; all values are median and interquartile range

Cytokine	Survivors	Non-survivors	p
Day1			
IL-23	5286 (1,851–13,179) ($n=38$)	2121 (1,305–5,527) ($n=13$)	0.07
IL-27	536 (241–972) ($n=34$)	708 (428–2,889) ($n=12$)	0.08
IL-18	10.257 (6,459–18,550) ($n=37$)	7758 (5,346–14,775) ($n=13$)	0.46
Day7			
IL-23	10.191 (4,782–24,261) ($n=41$)	5272 (995–9,114) ($n=8$)	0.03
IL-27	289 (107–542) ($n=39$)	200 (144–1,086) ($n=7$)	0.64
IL-18	11.996 (6,816–20,740) ($n=40$)	12.866 (8,729–16,724) ($n=8$)	0.87

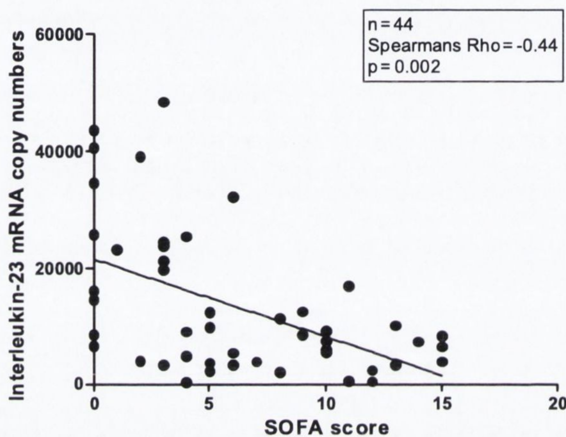

Fig. 2 Correlation between SOFA score and IL-23 mRNA levels on day 7 in the ICU group with severe sepsis or septic shock. IL-23 is expressed as copy numbers of cytokine mRNA per 10 million β-actin mRNA copy numbers. *SOFA*, Sequential Organ Failure Assessment

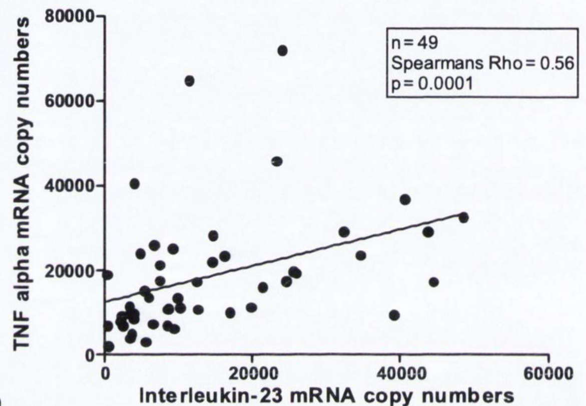

Fig. 3 Correlation between TNFα mRNA levels and IL-23 mRNA levels on day 7 in the ICU group with severe sepsis or septic shock. Cytokine mRNA levels expressed as copy numbers per 10 million β-actin mRNA copy numbers

($p=0.002$, Fig. 2). No association was detected between IL-27 mRNA levels and SOFA score. Similarly, IL-18 mRNA levels were not correlated with organ failure scores.

Mortality and mRNA levels

Seventeen of the 62 severe sepsis/septic shock patients (27%) died during the course of their ICU stay. On the first day of critical illness from the 52 patients with mRNA available for analysis 13 died prior to ICU discharge. There was a non-significant trend towards higher IL-23 mRNA levels ($p=0.07$) and lower IL-27 mRNA levels ($p=0.08$) in survivors than in non-survivors (Table 3). On the seventh day of critical illness 8 of the 49 severe sepsis/septic shock patients who had blood samples analysed for mRNA levels died prior to ICU discharge. These 8 non-survivors had lower IL-23 mRNA levels ($p=0.03$) than survivors (Table 3).

Change in mRNA levels over time

The severe sepsis/septic shock subgroup for whom blood samples were available at both time points were analysed for a change in mRNA levels over time. From day 1 to day 7 IL-18 mRNA levels decreased ($p < 0.0001$), IL-23 mRNA levels increased ($p=0.02$), and IL-27 mRNA levels remained unchanged. Quantitative data for these changes are presented in Appendix 3 (ESM).

Relationship to proinflammatory cytokines

We have previously described the relationship between proinflammatory cytokine mRNA levels (TNFα and IFNγ) and outcome in this cohort [3]. In the severe sepsis/septic shock group on day 1 there was a non-significant association between increased IL-23 mRNA and increased IFNγ mRNA levels ($n=51$, Spearman's $\rho=0.26$, $p=0.06$) and an inverse association between IL-27 and IFNγ mRNA

levels ($n = 44$, Spearman's $\rho = -0.29$, $p = 0.05$). In the severe sepsis/septic shock group on day 7 there was no relationship between IFNγ mRNA levels and the levels of IL-18, IL-23 or IL-27 mRNA. On day 7 of critical illness there was a significant correlation between IL-23 mRNA levels and TNFα mRNA levels ($p < 0.0001$, Fig. 3).

Discussion

In this study of patients with infection the presence of sepsis with associated critical illness and the mortality associated with this septic insult was linked to a distinct pattern of cytokine gene expression, specifically excess IL-27 and lesser IL-23 mRNA levels. There was no relationship between IL-18 mRNA and outcome or severity of illness.

Early work suggested that IL-23, which is produced by a wide range of cells, including dendritic cells, macrophages and mature T cells, exclusively acted with IL-12 to enhance IFNγ production [4]. However, by acting directly on macrophages to increase their production of TNFα, IL-23 has an additional role as an effector cytokine in innate immunity and, in a similar fashion to IL-12, provides an important link between innate and adaptive immunity [16]. Interestingly, in a multiple sclerosis model the IL-23 receptor was not present on naive T cells but was detectable on memory $CD4^+$ T cells [17]. This suggests that IL-23 is preferentially involved in sustaining cytokine production in the later stages of inflammation rather than initiating a pro-inflammatory response development from naive $CD4^+$ T cells. This may account for the association observed between IL-23 expression and SOFA scores in late sepsis that is not apparent in the early stages of the disease. Similarly, we observed a stronger relationship between deficient IL-23 expression and mortality in late sepsis in comparison to early sepsis.

More recently it has become clear that IL-23 has a major role in promoting inflammation through the induction of the IL-17 family of cytokines from a novel subset of $CD4^+$ T cells, termed Th17 cells [18]. It appears that IL-23 is necessary for the permanent differentiation of the Th17 lineage thus promoting sustained IL-17 production with potentially profound effects on inflammation and immune function [19]. The Th17 lineage of T cells have been purported to be of critical importance both in driving a variety of autoimmune inflammatory responses, including inflammatory bowel disease and animal models of collagen induced arthritis and experimental autoimmune encephalitis and also in host protection against bacterial infection [20–23]. Furthermore, while the interaction between the T cell subsets is complex, gene deletion studies suggest that IL-12 preferentially induces IFNγ production whilst IL-23 preferentially induces IL-17 production [18].

IL-27 is a cytokine, structurally related to IL-12, whose definitive function has proved more elusive. Originally reported to promote the differentiation of Th1 cells from naive human $CD4^+$ T cells by acting synergistically with IL-12 [6], subsequent data indicated a role in suppression of T cell responses [24]. Recently, using a gene knockout model, investigators have demonstrated that IL-27 regulates macrophage function and that the loss of IL-27 production is associated with a greater capacity to eliminate microbial infections with resultant improved survival [25]. In addition, IL-27 may act by repressing the pro-inflammatory $CD4^+$ Th17 response in vivo [26, 27], thereby acting in an antagonistic fashion to IL-23.

Our data in patients with infection also suggests opposing actions for IL-23 and IL-27. It is plausible that an imbalance between IL-23 and IL-27 gene expression in patients with infection contributes to an excess mortality by repressing protective inflammatory bactericidal responses by phagocytic cells. There are no prior studies of mRNA levels for these cytokines in humans with sepsis, and therefore although we have demonstrated an association between cytokine mRNA levels and adverse outcome with infection, it is not possible to determine directly from our data whether this link is reactive or causal. However, in animal models of sepsis, gene deletion studies indicate that IL-23 is protective whilst IL-27 impairs macrophage function [18, 28], and it is plausible that the pathophysiology is similar in humans.

Prior reports of disease-causing mutations in the p40 subunit shared by IL-12 and IL-23 and in the shared receptor subunit of IL-12 and IL-23, IL-12Rβ1, were originally attributed to resultant alterations in the IL-12/IFNγ response to infection [29–31]. Whilst it is currently appreciated that these mutations also affect IL-23 dependent pathways [32, 33] this is the first report confirming the potential importance of IL-23 gene expression in human sepsis in vivo. Although the importance of IL-23 as a mediator of pathogenic inflammation in inflammatory bowel disease is now appreciated, it had been suggested that IL-23 mediates only the local inflammatory processes whereas IL-12 is of paramount importance to the systemic inflammatory response [16, 34]. In contrast, we have demonstrated that systemic IL-23 mRNA levels accurately differentiates between groups based on the severity of response to infection. IL-12, when measured similarly, did not differentiate between these groups [3].

We observed that the effects of IL-23 and IL-27 were present both at the onset of sepsis, where cytokine levels differentiate between the three groups, and also in late sepsis, where we observed a strong association between IL-23 and TNFα mRNA levels. We have previously reported a linkage between deficient TNFα mRNA production in late sepsis and adverse outcome in this study population [3]. Taken together these data suggest that the deficient protective cytokine response in late sepsis is quite complex and possibly involves a number of inflammatory cell subpopulations. This study adds to the current available data supporting a vigorous pro-inflammatory response as being of paramount importance in containing

infection [1–3, 35]. However, as these cytokines are pleiotropic, it is unclear from these data whether this deficiency involves the innate or the adaptive system or possibly both, and this will require further study of cellular sub-populations to fully elucidate.

Previously IL-18 has been implicated as being causative in propagating sepsis induced organ failure by virtue of propagating an excessively vigorous pro-inflammatory response [36]. In contrast, we have previously described the benefits of a pro-inflammatory response in patients with sepsis [3]. Additionally, the current study demonstrates that IL-18 mRNA levels do not differentiate between critically ill infected patients and patients who tolerate infection with relative impunity and levels do not relate to severity of illness in the ICU population. Recent evidence from animal models supports this view [37]. Indeed it might even be inferred from the greater levels of IL-18 in bacteraemic patients that IL-18 is a protective cytokine produced in response to infection. However, as yet the role of IL-18 in human sepsis remains unclear.

Although protein quantification may ideally be the most accurate representation of gene function, we have previously demonstrated that protein analysis lacks sensitivity and accuracy in this setting [3]. This study again shows that QRT-PCR is a sensitive and accurate method of quantifying immunological mediators and has the potential for widespread use in a clinical scenario. It has been suggested that the lack of correlation between mRNA and protein can be explained by the technical imprecision of the methods used to determine the protein expression levels [38]. This is particularly problematic when dealing with genes expressed at relatively low levels such that commercially available kits have difficulty detecting protein product. Recently, however, investigators have reproducibly found positive correlations between mRNA and protein expressed at low levels using isotope coded affinity tags to quantify protein expression [11]. This implies that mRNA levels acts as an accurate surrogate of protein expression in this setting.

Additionally, our definition of septic shock differs slightly from consensus conference guidelines [12]. In the place of using systolic blood pressure as a guide to the commencement of inotropes, clinical practice in this ICU is to commence inotropes when the mean arterial pressure remains below 60 mmHg despite adequate fluid resuscitation. This approach is recommended by some publications [39]. In practice, however, the vast majority of our patients met the blood pressure requirements for both definitions of septic shock.

As supported by our data, we suggest that IL-18 antagonists are unlikely to prove of benefit in human sepsis. However, in patients with sepsis up regulation of a cytokine based immune response with exogenous IL-23 or with IL-27 antagonists in a timely and targetted fashion warrants further research.

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