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The Monocyte Protein C Pathway: Implications in Human Diseases

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A thesis submitted for the degree of Doctor of Philosophy

Department of Genetics

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January 2004

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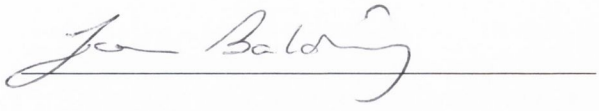

Joanna Balding

For Mum, Dad, Claire, and Lucy.

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Table 5.16 Genotype counts for cytokine polymorphisms in a control population and a stroke patient population

SUMMARY

The protein C (PC) pathway provides an important link between the coagulation, fibrinolytic, and inflammatory pathways. PC/activated protein C (APC) is one of very few therapies shown to effectively reduce the morbidity and mortality states seen in severe sepsis and septic shock. Originally identified as a naturally occurring anti-coagulant, PC has subsequently been shown to have potent anti-inflammatory properties. Given the significance of activated monocytes in the pathophysiology of severe sepsis and the recent discovery of expression of the endothelial protein C receptor (EPCR) on the monocyte cell surface, we investigated the effect of PC on the gene expression profile of the monocytic cell line THP-1. Microarray technology was used to determine the effect of PC on the expression levels of approximately ten thousand human genes in untreated and LPS activated THP-1 cells. Results were confirmed by investigating the expression of a number of genes included in the microarray by real-time PCR and ELISA. We showed that PC effectively inhibits the induction of all LPS induced genes, most notably many chemokines and inflammatory cytokines. However, PC did not have significant effect on gene expression levels in unstimulated monocytes, nor did it affect genes involved in apoptosis as previously reported in endothelial cells. In order to further investigate the mechanism by which PC inhibits LPS gene induction, we used flow cytometry and real-time PCR to study the monocyte cell surface and mRNA levels of TLR4, the main receptor and signal transducer for LPS. We also looked at the cell surface levels of CD14. The effect of PC and also of low molecular weight heparin (LMWH) was studied, as the latter, albeit an anti-coagulant, has putative anti-inflammatory properties which has been found effective in the treatment of inflammatory bowel disease. Despite a reduction in LPS-induced TLR4 levels by PC, we concluded that reduction of the LPS receptors is not the mechanism by which this protein exerts its anti-inflammatory effects. Observed effects on the levels of the LPS receptors TLR4 and CD14 was thought to be a result of the general inhibition of LPS-induced gene expression by PC. Finally, diseases involving inflammation, such as meningococcal septicaemia and inflammatory bowel disease, are relatively common in humans. The clinical relevance of seven previously documented polymorphisms in cytokine genes to five disorders involving excess inflammation was assessed using novel RFLP technology.

ABBREVIATIONS

A	adenine
AAMP	angio-associated migratory factor
AMV	avian myeloblastosis virus
ANOVA	analysis of variance
AP-1	activating protein-1
APC	activated protein C
ARDS	acute/adult respiratory distress syndrome
ARF-1	ADP-ribosylation factor 1
AT	antithrombin
ATF	activating transcription factor
ATT	α_1-antitrypsin
bp	base pair(s)
BSA	bovine serum albumin
C	cytosine
CPB	cardiopulmonary bypass
CD	Crohn's disease
CD14	cluster of differentiation antigen 14
cDNA	complementary (or copy) deoxyribonucleic acid
C/EBP	CCAAT/enhancer binding protein
CI	confidence interval
Con A	concanavalin A
CRE	cAMP response element
CREB	CRE-binding protein
DEPC	diethylpyrocarbonate
DIC	disseminated intravascular coagulation
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
dNTP	deoxynucleoside triphosphates (dATP, dCTP, dGTP, dTTP)
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EPCR	endothelial protein C receptor
ERK	extracellular-signal regulated kinase
FII-FXIII	Factor II to Factor XIII
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FSC	forward scatter characteristics
g	centrifugation force
G	guanine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPI	glycosylphosphatidyinositol
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMWK	high molecular weight kininogen
IBD	inflammatory bowel disease

ICAM	intercellular adhesion molecule
IFN	interferon
IL	interleukin
IL-1RA	interleukin 1 receptor antagonist
IRAK	IL-1R-associated kinase
IU	international units
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
kb	kilobase
kDa	kiloDalton
LMWH	low molecular weight heparin
LPS	lipopolysaccharide
MAC	membrane attack complex
MAPK	mitogen activated protein kinase
MCP-1	monocyte chemoattractant protein 1
MHC	major histocompatibility complex
MI	myocardial infarction
MOF	multiple organ failure
mRNA	messenger ribonucleic acid
MTHFR	methylenetetrahydrofolate reductase
NAC	no amplification control
NF-κB	nuclear factor kappa B
nm	nano metre
NTC	no template control
OD	optical density
PAF	platelet activating factor
PAI	plasminogen activator inhibitor
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PC	protein C
PCI	protein C inhibitor
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PHA	phytohaemagglutinin
PK	prekallilrein
PPD	purified protein derivative
PS	protein S
PsA	psoriatic arthritis
PTDSS-1	phosphatidylserine synthase-1
RA	rheumatoid arthritis
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
RPE	r-phycoerythrin
rpm	revolutions per minute
SAGE	serial analysis of gene expression
SD	standard deviation
SE	standard error
SIRS	systemic inflammatory response syndrome
SNP	single nucleotide polymorphism
SSC	side scatter characteristics
STAT	signal transducer and activator of transcription

T	tyrosine
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TGF	transforming growth factor
TLR	toll-like receptor
TM	thrombomodulin
TNF	tumour necrosis factor
U	units
UC	ulcerative colitis
UV	ultraviolet
VNTR	variable number tandem repeats

PUBLICATIONS

Balding J, Healy CM, Livingstone WJ, White B, Mynett-Johnson L, Cafferkey M, and Smith OP. (2003) Genomic polymorphic profiles in an Irish population with meningococcaemia: is it possible to predict severity and outcome of disease? *Genes Immun* **4** (8): 533-540.

Balding J, Kane D, Livingstone W, Mynett-Johnson L, Bresnihan B, Smith O, and FitzGerald O. (2003) Cytokine gene polymorphisms: association with psoriatic arthritis susceptibility and severity. *Arthritis Rheum* **48** (5): 1408-1413.

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CHAPTER 1

Introduction

1.1 INFLAMMATION OVERVIEW

Inflammation is a localised, protective response involving a series of cellular and chemical interactions. The inflammatory process is stimulated in response to mechanical or thermal trauma, necrosis, radiation, malignant growth, or microbial infection, and functions by limiting the spread of damaged tissue or an infectious agent. Chemicals are released from damaged tissue as a direct result of trauma or in response to toxin or other factors released from micro-organisms, and these initiate inflammation, which ultimately involves the concerted actions of the immune, kinin, fibrinolytic, and coagulation systems. The classic signs of inflammation, caused by the effects of cytokines on local blood vessels, include heat, pain, redness and swelling.

Inflammation usually refers to the local response at the site of tissue injury or infection. These local events include vasodilation, leukocyte influx at the site of injury stimulated by vascular endothelial cell expression of adhesion molecules, increased capillary permeability, increased blood flow, exudation of fluids, activation and differentiation of monocytes and macrophages, and enhanced coagulation activity. If the stimulus that induces the localised response is severe enough, a large range of systemic events also occur, which are collectively referred to as the acute phase response (APR) (Kushner, 1982; Koj, 1989; Baumann and Gauldie, 1994). These systemic events, which are induced by a group of inflammatory mediators, especially TNF-α, IL-1, IL-6, and IFN-γ, include neurological, endocrine, and metabolic changes. These changes are manifested as fever, altered synthesis of endocrine hormones, thrombocytosis, leukocytosis, alterations in lipid metabolism, changes in synthesis of hepatic plasma proteins (the acute phase proteins), and activation of the procoagulant, anticoagulant, kinin, and complement pathways. Although this acute phase response is thought to be of benefit in restoring homeostasis to a host that has been injured, an excessive production of these inflammatory mediators is known to have negative, sometimes lethal, effects such as those seen, for example, in septic shock.

Diseases involving inflammation are a major cause of mortality and morbidity in humans. Deficiencies in the inflammatory process can have severe consequences for a host, and

excessive, prolonged, or dysregulated inflammation, or recognition of self tissue as foreign, contributes to the pathogenesis of trauma, autoimmune diseases, and infection. Such a response may lead to a range of inflammatory diseases including, amongst others, inflammatory bowel disease (IBD), type I diabetes mellitus, Alzheimer's disease, multiple sclerosis, atherosclerosis, rheumatoid arthritis, psoriasis, systemic lupus erythematosus, neisserial or pneumococcal meningitis, mucocutaneous leishmaniasis, and systemic inflammatory response syndrome (SIRS). An understanding of the regulation of the inflammatory process is vital in developing new methods of prevention and treatment for these diseases. However, many of the processes involved in the inflammatory response remain poorly understood, and the underlying mechanisms that cause inappropriate immune responses are in the main unknown.

1.2 MEDIATORS OF THE INFLAMMATORY RESPONSE

The inflammatory response is initiated by a complex series of interactions involving a variety of chemical mediators whose interactions are still only partly understood. The chemical mediators involved in the inflammatory response are derived from a variety of sources. Some are produced by invading micro-organisms (exogenous mediators), whilst others are derived from the immune system itself (endogenous mediators), and are produced by damaged cells that are responding to tissue injury, by the multiple white blood cell types, and by the various plasma enzyme systems. Endogenous mediators can be divided into those derived from plasma and those derived from cells.

1.2.1 Plasma Derived Mediators

Plasma contains at least four enzymatic pathways that play an important role in haemostasis and the regulation of inflammation: the complement, kinin, fibrinolytic, and coagulation systems. These systems are inter-related and produce various important mediators of the inflammatory system. Figure 1.1 shows some of the links between the four enzymatic systems discussed in this section.

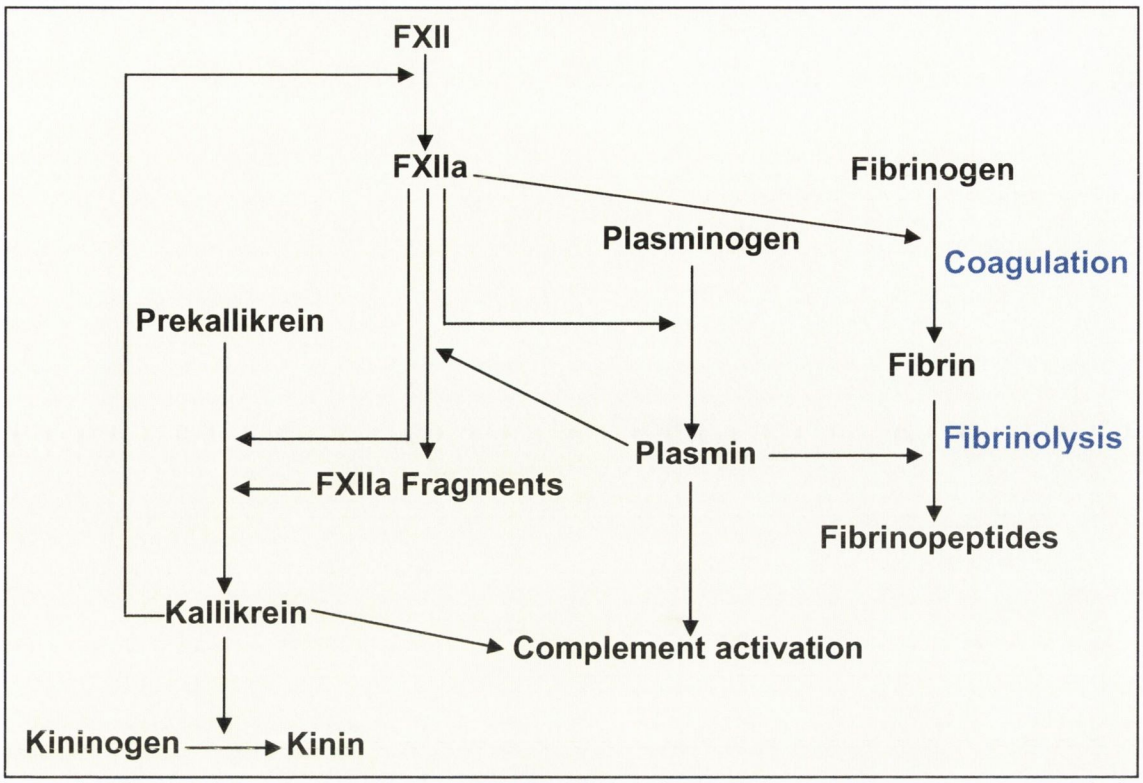

Figure 1.1 Interactions that occur between the kinin, fibrinolytic, complement, and coagulation pathways. FXII = factor XII; FXIIa = activated factor XII.

The Kinin System

The kinins, bradykinin and *lysyl*bradykinin, are small peptides (9-11 amino acids) that are present in blood plasma in the form of precursor molecules, kininogens. These are activated by various proteases, collectively known as kininogenases. Kininogenases are produced by hepatocytes and act as part of the acute phase response, as cofactors in the coagulation system, and as inhibitors of cysteine protease enzymes in addition to releasing kinins. Kinins are activated when tissue damage occurs and cause increased capillary permeability and vasodilation, contraction of smooth muscle, and stimulation of arachidonic acid metabolism.

The most important vascular permeability factor is bradykinin, production of which is initiated by the coagulation factor XII (FXII), also known as Hageman factor. This converts prekallikrein (PK) to kallikrein (a kininogenase) which then cleaves kininogen to produce bradykinin, a potent vasoactive peptide and a stimulator of pain receptors at the site of infection. Bradykinin activity and generation is carefully controlled internally by inhibitors such as complement factor 1 (C1) and α_2-macroglobulin as various stages in the activation cascade. A number of plasma enzymes also control bradykinin activity, for example carboxypeptidase N, which removes its C-terminal arginine residue, and thus

inactivates the peptide. The production of *lysyl*bradykinin from prokallikrein is mediated by tissue kallikrein.

The Complement Cascade

Complement is a complex system involving more than 30 glycoproteins that are present in serum as components, factors, regulators, and on the surface of different cells as receptors. The complement system is present in blood in an inactive state, and can be stimulated by a variety of agents including enzymes released from dying cells during tissue necrosis, antigen-antibody complexes formed during infection, endotoxins of Gram-negative bacteria, and by products of the kinin, coagulation and fibrinolytic systems. It is activated by one of two pathways: the classical pathway (activation by immune complexes), or the alternative pathway (activation by other substances, mainly of bacterial origin).

The complement system involves a cascade of proteolytic cleavage events where each of the components is cleaved into two fragments, one of which participates further in the cascade, the *b* fragment, whilst the other modifies the inflammatory response, the *a* fragment (Figure 1.2). The components of the complement system are termed C1 to C9. Both pathways share a common final pathway from C3 to the formation of a unit known as the membrane attack complex (MAC). This unit, inserted in the membrane, acts as a channel allowing fluid and molecule transfer in and out of the cell. Other important components of the complement system include anaphylatoxins C3a, C4a, and C5a. C5a is chemotactic for neutrophils, increases vascular permeability, and releases histamine from mast cells and basophils (degranulation); C3a has similar properties to, but is less active than, C5a. The production of anaphylatoxins follows not only from complement activation, but also from activation of other enzyme systems which may directly cleave C3, C4, and C5. Such enzymes include plasmin, kallikrein, tissue and leukocyte lysosomal enzymes, and bacterial proteases. Other components of the complement system include C4b, C2a, and C3b, which facilitate opsonisation of bacteria, leading to phagocytosis.

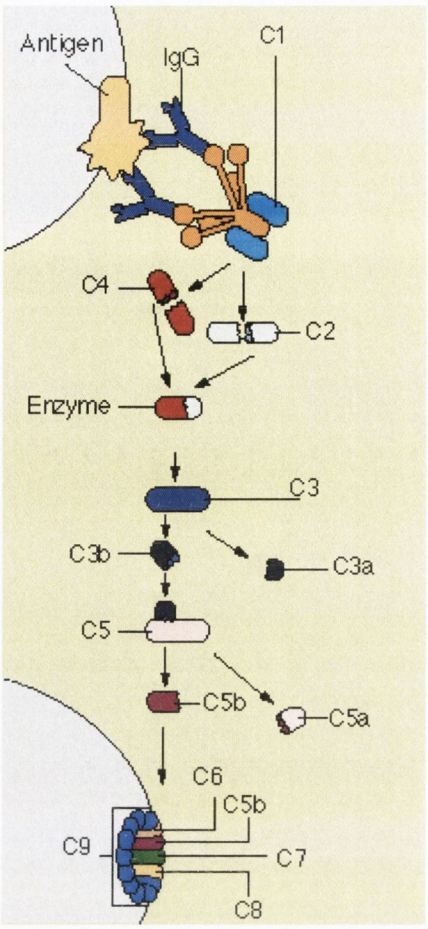

Figure 1.2 The complement system (from http://press2.nci.nih.gov/sciencebehind).

The Coagulation Cascade

The coagulation pathway, like the complement system, is a proteolytic cascade that was initially recognised in 1964 (MacFarlane, 1964; Davie and Ratnoff, 1964). It is responsible for the conversion of soluble fibrinogen into fibrin, which forms a clot. The fibrin clot closes off the area of injury from the rest of the body, helping to prevent a spread of the tissue damage or of the infection, and preventing blood loss after injury. This system mediates the inflammatory process on several levels due to interaction of its components with the other plasma enzyme systems. For example, FXII, once activated by contact with extracellular materials such as basal lamina and various proteolytic enzymes of bacterial origin, can activate the coagulation, kinin and fibrinolytic systems.

Plasma coagulation factors are glycoproteins, mainly produced in the liver, that circulate in the blood as inactive proteins which only undergo conversion to active enzymatic or co-factor forms after vessel damage. The Roman numeral method for naming clotting factors reflects the order of identification rather than the place in the cascade. The coagulation cascade can occur *via* one of two pathways. The tissue factor (TF) pathway (historically the extrinsic pathway) is activated as a result of exposure of blood to TF on the surfaces of

cells in the extravascular space, and provides a rapid response to tissue injury. This pathway is now considered the principal initiator of blood coagulation and is activated during normal haemostasis. The contact (intrinsic) pathway is initiated when blood comes into contact with sub-endothelial tissues that are exposed as a result of tissue damage, or with a negatively charged surface. This pathway was initially thought to be activated as a result of the convergence of FXII, prekallikrein, high molecular weight kininogen (HMWK), and FXI at the activating surface (the 'contact system'). However, it has recently emerged that FXI can be activated by thrombin (FIIa), even in the absence of a negatively charged surface (Gailani and Broze, 1991; Naito and Fujikawa, 1991). Both TF and contact pathways converge at the activation of FX and a common pathway involving the generation of thrombin (FIIa) from prothrombin (FII) leads ultimately to the conversion of soluble fibrinogen to insoluble fibrin. The main steps involved in the coagulation cascade are shown in Figure 1.3.

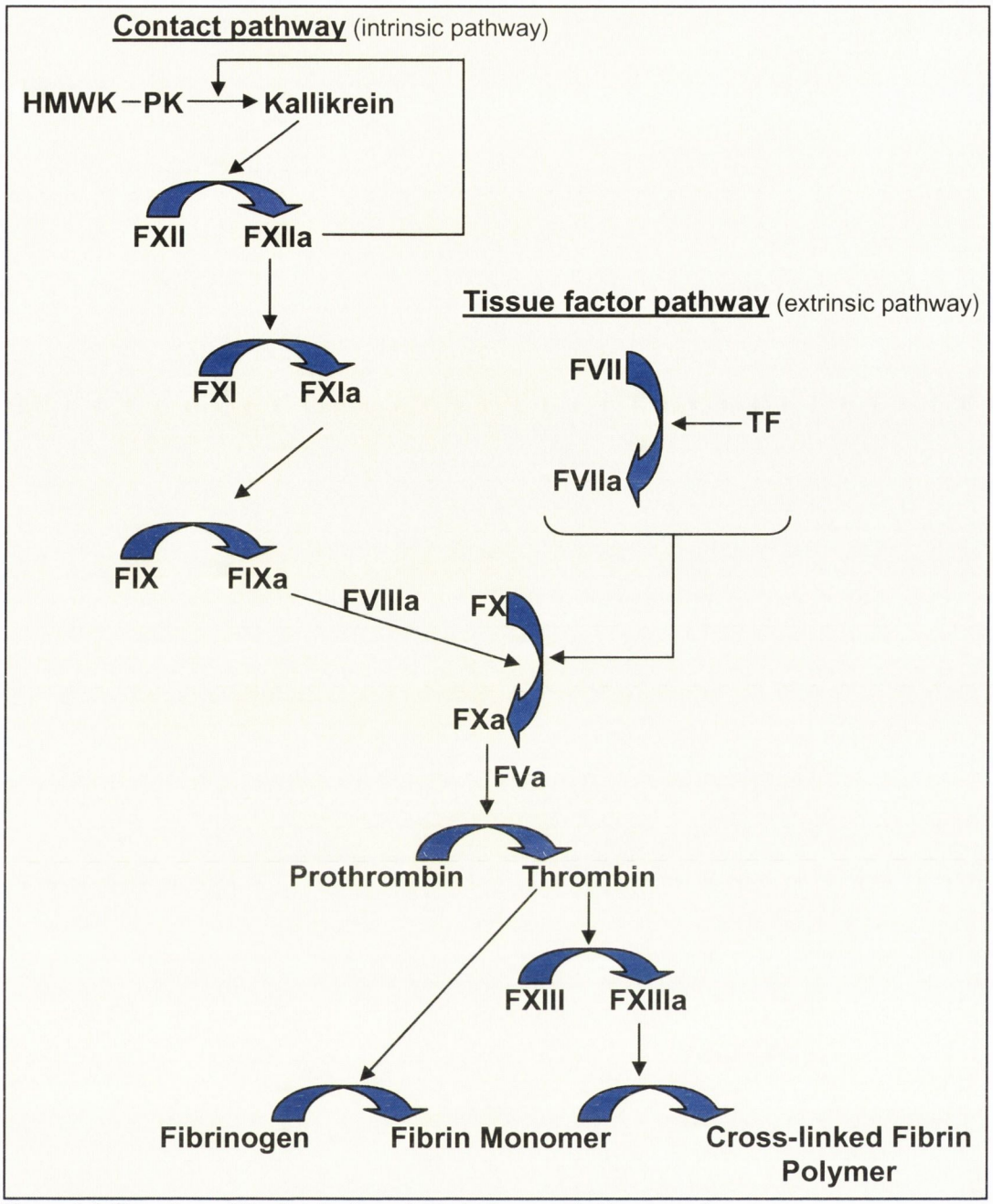

Figure 1.3 Overview of the intrinsic and extrinsic coagulation cascades.

The Fibrinolytic System

The fibrinolytic system, responsible for clot removal once haemostasis is restored and the tissue is repaired, comprises a system of initiators, co-factors, feedback mechanisms and inhibitors. Plasmin, responsible for the lysis of fibrin into fibrin degradation products (FDPs) and D-dimers, circulates as inactive plasminogen (Castellino, 1984), which is activated by urokinase-type plasminogen activator (uPA) or tissue-type plasminogen activator (tPA) (Astrup, 1966). Fibrinolysis can also be activated by agents present in the blood such as FXIIa and PK. Cleavage of non cross-linked fibrin by plasmin results in FDPs, which inhibit platelet aggregation, the action of thrombin on fibrinogen, and the

digestion of cross-linked fibrin yields D-dimers. Inhibitors, the most important of which are plasminogen activator inhibitor 1 (PAI-1) and plasmin-α2-antiplasmin (PAP), tightly control the generation and activity of plasminogen. Plasmin also activates the complement system and can split FXIIa into two fragments which activate PK (see Figure 1.1).

1.2.2 Cell Derived Mediators

Histamine, a chemical released by a variety of cells including mast cells, basophils, eosinophils and platelets in response to tissue injury, causes vasodilation and increased permeability by binding to receptors on capillaries and venules and causing small temporary gaps between adjacent endothelial cells, thus permitting enzymes involved in the coagulation system to enter the damaged tissue. Serotonin (5-hydroxytryptamine), also capable of increasing vascular permeability, is present in high concentration in platelets and mast cells. Prostaglandins and leukotrienes are a group of long-chain fatty acids derived from arachidonic acid and synthesised upon stimulation by many cell types including neutrophils and macrophages. They cause increases in vascular permeability and affect platelet aggregation as well as promoting neutrophil activation and chemotaxis. Lysosomal compounds are released from neutrophils and include cationic proteins, which can affect vascular permeability, and neutral proteases, which can activate complement. Lymphocytes release chemical messengers known as lymphokines which can have vasoactive and chemotactic properties. Platelet activating factor (PAF) is produced by many different kinds of stimulated cells including basophils, neutrophils, monocytes, macrophages, and endothelial cells. It is 100-to-10 000 times more potent than histamine with respect to its vasoactive properties. It also stimulates other cells to increase their functional and metabolic activities, is a mediator of platelet aggregation and degranulation, and mediates interaction between leukocytes and endothelial cells.

1.2.3 Cytokine Regulation of the Inflammatory Response

An effective immune response involves the activities of inflammatory cells, lymphoid cells, and haematopoietic cells. The interactions between these cells are mediated by a group of chemicals known as cytokines. Although a variety of cells can produce cytokines, the principal producers are macrophages and T-helper (T_H) cells. Cytokines produced from these cells can then activate a network of interacting cells. In addition, cytokines often

induce the synthesis of other cytokines, resulting in a cascade of cytokine activity. The network of cytokine effects is illustrated in Figure 1.4.

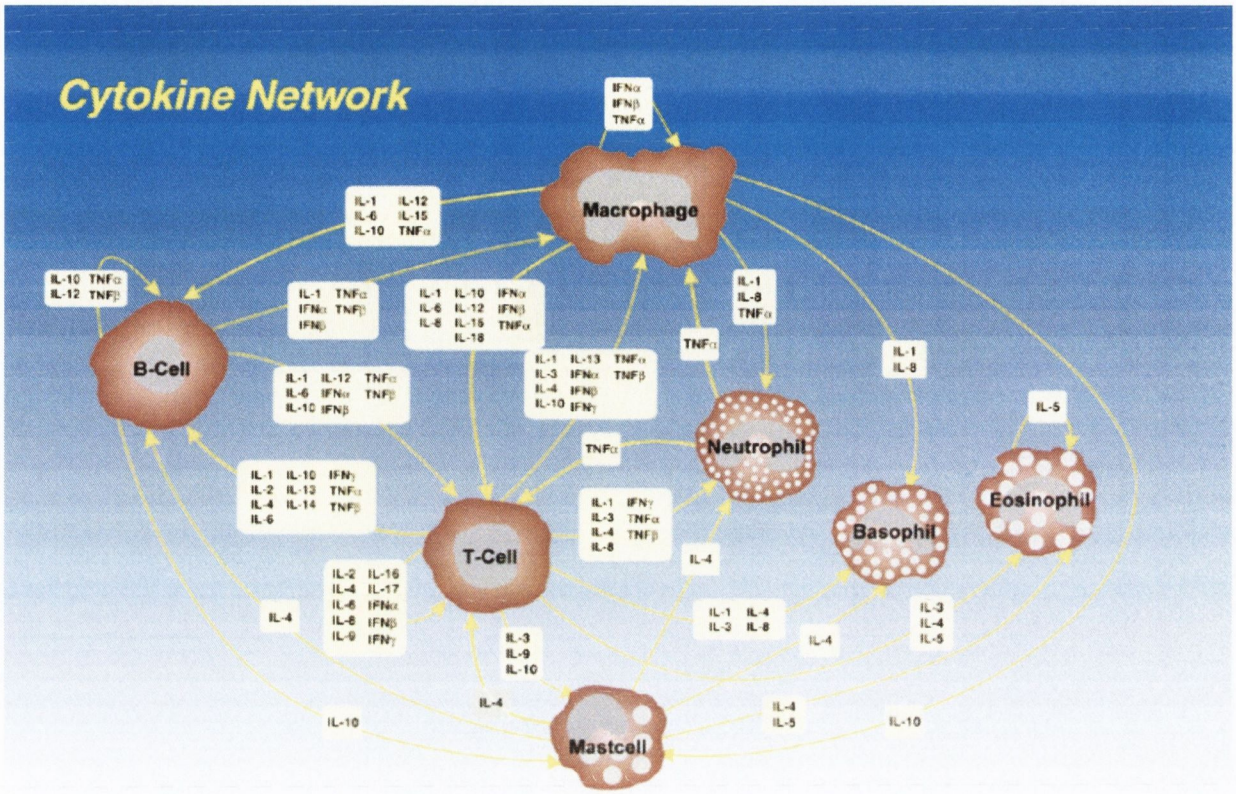

Figure 1.4 The cytokine network (Sinclair, 2000).

Cytokines can be divided into those involved in the acute inflammatory response and the chronic inflammatory response (Figure 1.5) (Feghali and Wright, 1997). Of those involved in the acute inflammation, IL-1 and TNF-α are known to be the principle cytokines involved, acting as potent inflammatory molecules inducing many of the local and systemic effects observed in the inflammatory response.

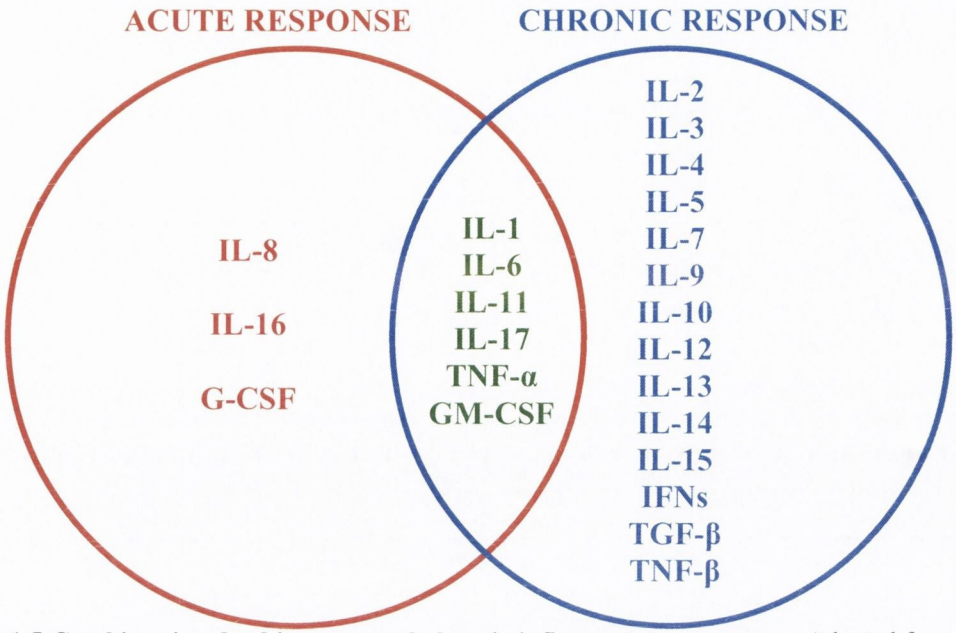

Figure 1.5 Cytokines involved in acute and chronic inflammatory responses. Adapted from (Feghali and Wright, 1997).

From the point of view of inflammation, cytokines are generally categorised as proinflammatory or anti-inflammatory (Table 1.1). Proinflammatory cytokines are produced predominantly by activated macrophages and are involved in the up-regulation of inflammatory reactions. Anti-inflammatory cytokines are generally T cell-derived and are involved in the down-regulation of inflammatory reactions (Stvrtinova *et al*, 1995).

Table 1.1 Groups of pro-inflammatory and anti-inflammatory cytokines. Adapted from (Stvrtinova *et al*, 1995).

Group	Cytokines
Pro-inflammatory:	
• Up-regulation during inflammation	IL-1, TNF-α, IL-6, IFN-α, IFN-β, chemokines
• Stimulation of acute phase reactants	IL-1, IL-6, IL-11, TNF-α, IFN-γ, TGF-β, LIF, OSM, CNTF
• Chemoattractants:	
- CXC chemokines	IL-8, PF-4, PPBP
- CC chemokines	MIP-1α, MIP-1β; MCP-1, MCP-2, MCP-3, RANTES
- C chemokines	lymphotactin
• Stimulation of pro-inflammatory cytokines	IL-12
Anti-inflammatory:	
• Inhibition of production of pro-inflammatory cytokines	IL-4, IL-10, IL-13

LIF = leukaemia inhibitory factor; OSM = oncostatin M; CNTF = ciliary neurotrophic factor; PF-4 = platelet factor 4; PBP = pro-platelet basic protein; MIP = macrophage inflammatory protein; MCP = monocyte chemoattractant protein; RANTES = regulated upon activation, normally t-expressed, and presumably secreted.

Cytokines are small glycoproteins that are generally not expressed unless stimulated by specific factors such as infection and inflammatory products, UV light, heat shock, or hyperosmolarity. They are pleiotropic and elicit their effects in a paracrine or autocrine manner (Feghali and Wright, 1997). Cytokines act non-enzymatically by binding to high affinity cell surface receptors on target cells. Most of these receptors fall into one of two main families: receptor tyrosine kinases (RTKs) and receptors which trigger a Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway (Aringer *et al*, 1999; Hubbard, 1999) .

RTKs are triggered by such ligands as insulin, vascular endothelial growth factor (VEGF), macrophage-colony stimulating factor (M-CSF), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) (Hubbard, 1999). When the ligand binds to two adjacent receptors an active dimer is formed which is a tyrosine kinase. This attaches phosphate groups to tyrosine residues on itself and on other proteins, converting them to an active state. Many of these other proteins are also tyrosine kinases, thus causing a cascade of expanding phosphorylation events in the cytosol. Some of the cytosolic tyrosine kinases act by entering the nucleus and transferring their phosphate groups to transcription factors, thus activating them and ultimately leading to alterations in gene transcription, whilst others act indirectly through secondary messengers. RTK signalling is turned off by receptor mediated endocytosis, where the receptor complex is engulfed and destroyed.

JAK-STAT pathways are triggered by many ligands, including interferons, most of the interleukins, growth hormone, prolactin, erythropoietin, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Aringer *et al*, 1999). Ligand binding causes two receptors to form a dimer which activates a JAK protein, found associated with the receptors on the cell surface. The activated JAK phosphorylates tyrosine residues on STAT proteins which then also form dimers and enter the nucleus where they bind to specific sequences in gene promoters, affecting transcription levels. JAK-STAT pathways are shorter than those triggered by RTKs and thus produce a more rapid response by cells to the ligands.

Other groups of cytokine receptors include the transforming growth factor-β (TGF-β) receptors which, when they bind their ligand (such as TGF-β, activin, bone morphogenetic proteins), become kinases that attach phosphate groups to serine and/or threonine residues in the target proteins. The TGF-β receptors signal through phosphorylation of Smad

proteins in the cytosol (Shi and Massague, 2003). The TNF/nerve growth factor (NGF) receptor family counts amongst its members the TNF receptors that are bound by TNF-α and TNF-β (Wallach *et al*, 1999). Most biological activities are mediated through the TNF type I receptor, although a type II receptor exists. Ligand binding causes trimerisation of receptors leading to activation of apoptosis, nuclear factor-κB (NF-κB), and c-Jun N-terminal kinase (JNK) (Wallach *et al*, 1999). The IL-1 receptor/Toll-like receptor (IL-1R/TLR) superfamily is defined by a homologous TIR (Toll/IL-1 receptor) domain (Medzhitov *et al*, 1997). The TLRs, which respond to various microbial products, are discussed later in this chapter. The IL-1R and IL-18R also belong to this family of receptors which signal through the adaptor protein MyD88 and IRAK which becomes phosphorylated, leading to the activation of NF-κB.

Tumour Necrosis Factor-alpha

TNF-α, the most abundant product of activated macrophages, is believed to be one of the main pro-inflammatory cytokines, playing a central role in the initiation and regulation of the cytokine cascade involved in the inflammation response. Along with well-characterised pro-inflammatory effects, TNF has many other diverse activities including signals for cellular differentiation, proliferation, and death. Although it shares a range of pleiotropic activities with other cytokines, for example IL-6 and IL-1, TNF is the only cytokine capable of triggering the complete systemic response that defines septic shock syndrome (Tracey *et al*, 1988; Tracey, 1991).

The *TNF* locus, situated in the central Major Histocompatibility Class (MHC) region, contains genes coding for TNF-α and TNF-β, amongst others. These cytokines bind to the same TNF receptors (two types) which are expressed on virtually all somatic cells. Human TNF-α is synthesised as a pro-hormone, which is anchored to the cell surface. The secreted form of TNF-α is then generated by proteolytic cleavage of the transmembrane portion of the molecule. Both secreted and transmembrane forms of the cytokine are active (Perez *et al*, 1990). TNF-β exists as a secreted form only (Paul and Ruddle, 1988). TNF-α and TNF-β have approximately 30% homology at the amino acid level, but since they bind to the same receptors, they share many biological activities.

Although activated monocytes and macrophages are the main producers, TNF-α secretion can be induced in many cell types including lymphocytes, smooth muscle cells, astrocytes,

polymorphonuclear leukocytes, NK cells, mast cells, and others. Along with IL-1β, TNF-α is one of the earliest cytokines to be produced by activated monocytes/macrophages. It then stimulates the release of IL-6, macrophage inflammatory protein-1 (MIP-1), macrophage migratory inhibitory factor (MIF), and other pro-inflammatory cytokines (Dudley *et al*, 1995; Hirokawa *et al*, 1997). Circulating TNF-α protein is not usually detectable in healthy humans. However, monocytes secrete TNF-α in response to infectious challenge (Burchett *et al*, 1988). TNF-α synthesis is stimulated in monocytes and macrophages by exogenous substances such as LPS, by bacterial exotoxins like staphylococcal superantigens, or by endogenous mediators such as IL-1. Inhibition of TNF-α production is mediated by IL-4, IL-10, and TGF-β (Espevik *et al*, 1987; te Velde *et al*, 1990; Marchant *et al*, 1994).

TNF-α plays an important role in host defence, but can cause severe damage when produced in excess or when the pro- and anti-inflammatory cytokine balance is upset. High levels of TNF-α are found in a variety of infectious and inflammatory states. Anti-TNF-α therapy has been found to be beneficial in both Crohn's disease and in rheumatoid arthritis (Elliott *et al*, 1994; Neurath *et al*, 1997). TNF-α has been found to contribute to neurovascular lesions in cerebral malaria, and early hepatic necrosis in virus-induced liver diseases. This pro-inflammatory cytokine is also present at high levels in bacterial meningitis and in sepsis syndrome, with higher levels correlating with severity of disease and adverse outcome (Waage *et al*, 1987; Tracey, 1991; Westendorp *et al*, 1995).

Interleukin-1

The IL-1 gene family, a cluster located on chromosome 2, contains three members: *IL1α*, *IL1β*, and *IL-1RN* (encoding the IL-1 receptor antagonist protein). IL-1α and IL-1β, both agonists, are potent proinflammatory cytokines (Dinarello and Wolff, 1993), whereas IL-1 receptor antagonist (IL-1Ra) inhibits the activities of IL-1. This naturally occurring antagonist appears to be a unique feature in cytokine biology (Dinarello, 1991).

The two forms of IL-1, IL-1α and IL-1β, are indistinguishable in terms of biological activity. They are structurally related at the three-dimensional level and bind to the same cell-surface receptors despite the fact that they are products of separate genes and are different in their amino acid sequence (March *et al*, 1985). Both forms of IL-1 are synthesised by mononuclear phagocytes when stimulated by bacterial toxins or

inflammatory mediators. IL-1α is thought to be a regulator of intracellular events, and a mediator of local inflammation, as this protein is synthesised in association with cytoskeletal microtubules, not in the endoplasmic reticulum like IL-1β, and therefore mostly remains in the cytosol (Stevenson *et al*, 1992). On the other hand, IL-1β is generally thought of as an intercellular mediator, since it is released by the monocyte into the extracellular space, having been cleaved to become the mature form of IL-1β by interleukin-1β-converting enzyme (ICE) (Thornberry *et al*, 1992).

Both IL-1α and IL-1β mediate their activities through the IL-1 receptor *I* (IL-1R*I*), one of two members of the IL-1 receptor family, found on a variety of cells including monocytes, endothelial cells, epithelial cells, hepatocytes, fibroblasts, keratinocytes, T-cells, and neutrophils. The other member, IL-1R*II*, binds IL-1 but does not transduce a signal. It therefore acts as a sink for IL-1β, and is referred to as a decoy receptor (Colotta *et al*, 1994). When IL-1R*I* is occupied by IL-1Ra, biological functions of IL-1 are blocked, since signal transduction cannot occur (Dripps *et al*, 1991). The balance between IL-1 and IL-1Ra is of importance in normal and disease states, as the margin between beneficial levels of IL-1 and toxicity is very narrow in humans. The production of this antagonist by monocytes, fibroblasts, neutrophils, and macrophages is stimulated by IL-4, IL-6, IL-10, IL-13, GM-CSF, and TGF-β (Vannier *et al*, 1992; Wahl *et al*, 1993; Muzio *et al*, 1994).

IL-1β is generally undetectable in resting cells. Monocytes are the main source of secreted IL-1β, although it can also be produced by macrophages and peripheral neutrophil granulocytes. Expression of IL-1β can be enhanced by other cytokines and growth factors such as TNF-α, IFN-γ, IL-6, GM-CSF, and also by IL-1 itself. Phorbolesters, bacterial endotoxins, viruses, mitogens, and antigens are also potent stimulators of IL-1β. It is a multifunctional cytokine that affects most cell types, often along with another proinflammatory cytokine, TNF-α. Production is stimulated early in the acute phase response, and IL-1 acts to increase the expression of a variety of cytokine genes as well as a number of transcription factor genes. Treatment of monocytes with IL-1β stimulates the production of IL-1β, IL-6, TNF-α, and IL-8, presumably by stimulating the activation of transcription factors such as NF-κB and AP-1 (Tuyt *et al*, 1997). IL-1 is thought to play a pathogenic role in several diseases including sepsis, IBD, rheumatoid arthritis, leukaemia, psoriasis, and diabetes. Reduction of its production or blocking of its action is therefore a possible strategy for treating patients with such diseases (Dinarello and Wolff, 1993).

Interleukin-6

IL-6 is characterised by pleiotropy and redundancy of action. It has been shown to have both pro- and anti-inflammatory properties, and procoagulant properties (Stouthard *et al*, 1996; Tilg *et al*, 1997), is a main cytokine in the hepatic acute phase response, and plays an important role in the immunological cascade. The biological activities of IL-6 include stimulation of B cell differentiation, T cell proliferation, immunoglobin secretion, and hepatocytic acute phase protein production (Hirano, 1992). This cytokine is produced by a range of different cell types, including monocytes and macrophages, endothelial cells, fibroblasts, T-cells, and B-cells. IL-6 mRNA is constitutively expressed in many cell types at very low levels, but expression is induced during infection, trauma or immunological challenge in almost all tissues.

In monocytes and macrophages, production of IL-6 is stimulated by LPS, IL-6, IFN-γ, TNF-α, GM-CSF, PDGF, IL-1, CSF-1, viruses, adherence muramyl dipeptide (MDP), C5a, thrombin, and various other stimuli (Content *et al*, 1985; May *et al*, 1986; Helfgott *et al*, 1987; Kohase *et al*, 1987; Sanceau *et al*, 1989; Smith *et al*, 1992; Akira *et al*, 1993; Chomarat *et al*, 1995; Johnson *et al*, 1998). Inhibition of IL-6 production in monocytes can be caused by IL-4, IL-10, IL-1Ra, IL-13, and TGF-β (Gibbons *et al*, 1990; Musso *et al*, 1990; te Velde *et al*, 1990; Minty *et al*, 1993; Chomarat *et al*, 1995; Takeshita *et al*, 1996). The IL-6 receptor (IL-6R) is a member of the haematopoietic cytokine receptor family. The IL-6R complex comprises two functionally different membrane proteins: a ligand-binding chain (IL-6R) and a non-binding but signal transducing chain (gp130). The receptor is undetectable on a majority of lymphocytes but is expressed on monocytes, T cells, and peripheral blood mononuclear cells (Bauer *et al*, 1989; Hirata *et al*, 1989).

An increase in levels of IL-6 has been reported in several diseases, including systemic lupus erythematosus (SLE) (Linker-Israeli *et al*, 1991), IBD (Stevens *et al*, 1992), Castleman's disease (Yoshizaki *et al*, 1989), multiple myeloma (Dao *et al*, 1994), and systemic-onset juvenile chronic arthritis (Fishman *et al*, 1998). The cytokine has also been found to be a marker for poor outcome in sepsis. It has been found that levels of IL-6, along with IL-1Ra, remain very high in patients with severe septic shock, in contrast to levels of TNF-α and IL-1β which, although correlating with severity of shock and organ failure, decrease towards normal levels after 24 hours (Gardlund *et al*, 1995). The highest levels of IL-6 and IL-1Ra are also found in the most critically ill patients. The prognostic

value of IL-6 has also been shown in other studies (Hack *et al*, 1989; Waage *et al*, 1989). However, the involvement of IL-6 in the pathogenesis of septic shock could be limited, as demonstrated by the fact that monoclonal antibodies to IL-6 or its receptor in various murine models do not have an effect on outcome (Libert *et al*, 1992). IL-6 may therefore act as a marker, reflecting the magnitude and persistence of cellular damage in septic shock, whereas TNF-α and IL-1β may mediate initial septic tissue damage.

Interleukin-8

IL-8 was discovered in 1987 from LPS-stimulated monocyte culture supernatants (Yoshimura *et al*, 1987). The *IL-8* gene is part of a gene superfamily located on chromosome 4, sharing structural and functional homology with other members of the family. IL-8 is produced by activated monocytes/macrophages, neutrophils, and endothelial cells, and its release is stimulated by a range of factors including TNF-α, IL-1β, IL-2, mitogens, and viral/bacterial antigens (Mukaida *et al*, 1998). IL-8 is part of a group of mediators known as chemokines which have the ability to recruit inflammatory cells to sites of injury and inflammation. Its main inflammatory impact lies in its ability to act on neutrophils causing chemotaxis and neutrophil degranulation (Walz *et al*, 1987). Along with IL-1 and TNF, IL-8 is also involved in neutrophil recruitment by upregulating cell-surface adhesion molecule expression (Detmers *et al*, 1990), thus enhancing neutrophil adherence to endothelial cells. IL-8 has many other effects on neutrophils (Mukaida *et al*, 1998), as well as effects on basophils and eosinophils (White *et al*, 1989). However, the pathophysiological relevance of these effects on basophils and eosinophils remains to be determined. IL-8 exhibits selective chemotactic activities in that it chemoattracts neutrophils, basophils (White *et al*, 1989), and T lymphocytes (Larsen *et al*, 1989), but not monocytes/macrophages (Mukaida *et al*, 1998).

Chemokines are subdivided into four subfamilies, CXC, CC, C, CX3C, depending on the spacing of highly characteristic cysteine residues in their amino-terminal position (Mukaida *et al*, 1998). IL-8, one of a family of thirteen CXC chemokines, exhibits potent chemotactic activity for neutrophils like many chemokines in this group, while CC chemokines, such as monocyte chemotactic and activating factor/monocyte chemoattractant protein (MCAF/MCP-1), are involved in the activation of monocytes, macrophages and, lymphocytes (Mukaida *et al*, 1998).

Chemokines have been implicated in several inflammatory disorderss from acute neutrophil-mediated conditions such as acute respiratory distress syndrome (ARDS) to allergic asthma, arthritis, psoriasis, and chronic inflammatory disorders. IL-8 can be detected in synovial fluid from patients with various inflammatory rheumatic diseases (Partsch *et al*, 1997), and mucosal levels of IL-8 are elevated in patients with active ulcerative colitis (Nielsen *et al*, 1997; Ina *et al*, 1997).

Interleukin-10

IL-10 is produced by monocytes/macrophages, T-lymphocytes, B cells, and eosinophils (de Waal Malefyt *et al*, 1991; Yssel *et al*, 1992). In monocytes, IL-10 mRNA expression and protein secretion is seen when cells are stimulated with LPS, although this expression is delayed relative to that of TNF-α, IL-6, and IL-1β (Donnelly *et al*, 1995). This anti-inflammatory cytokine inhibits production of a wide range of cytokines in a variety of cell types. For example, IL-10 has been shown to inhibit the synthesis, at the transcriptional level, of IL-1β, IL-6, IL-8, GM-CSF, G-CSF, and TNF-α by activated monocytes and macrophages, whilst increasing levels of IL-1Ra, as well as having auto-regulatory effects (de Waal Malefyt *et al*, 1991; Fiorentino *et al*, 1991; Wang *et al*, 1994). It is thought that IL-10 acts to inhibit these cytokines by selective inhibition of NFκB activation in LPS-stimulated human monocytes (Wang *et al*, 1995). NFκB is an important transcription factor involved in the expression of inflammatory cytokines, and so IL-10 appears to have important deactivation effects on stimulated monocytes. IL-10 has also been shown in *in vivo* studies to protect mice against the lethal effects of endotoxin and improve survival (Howard *et al*, 1993; Kato *et al*, 1995), and administration of anti-IL-10 antibody worsens mortality (van der Poll *et al*, 1995).

1.3 CELLS OF THE INFLAMMATORY RESPONSE

A rapid response to inflammation requires the activities of a range of immune effector cells, collectively known as leukocytes. The average amount in human blood and main function of these cells are shown in Table 1.2. Mast cells are present in tissue and are therefore not included in this table, however they are classed as leukocytes and are thought to be the 'first responders' to infection or tissue trauma when they release vasodilatory and chemoattractant substances. Other types of leukocytes are subsequently recruited to the site of inflammation. The classes of leukocytes recruited can depend on the nature of inflammatory stimulant and the stage of the inflammatory response. Basophils and

eosinophils are more abundant when inflammation has been triggered by an allergic reaction or by parasites. Neutrophils are the primary cells to be recruited and thus are important in the acute phase response, whilst mononuclear cells, macrophages and lymphocytes, are the predominant cells in the subacute and chronic phase of the inflammatory response. Figure 1.6 gives a general view of the activation and functions of various cells involved in the immune response to infection.

Table 1.2 Leukocytes (white blood cells - WBCs) in normal human blood (average total WBCs = 7800/µl blood, generally between 4000 and 10000/µl blood).

Cell Type	Percent of total WBCs in blood	Function
Granulocytes (polymorphonuclear):		
- Neutrophils	50-70%	Earliest phagocyte recruited to site of infection. Granules containing toxins fuse with phagosomes to kill contents.
- Eosinophils	1-4%	Cytotoxic – release highly toxic granular contents (proteins/free radicals) that kill pathogens.
- Basophils	0.5-1%	Discharge contents of granules releasing histamine, serotonin, prostaglandins, leukotrienes (vasoactive substances).
Agranulocytes (mononuclear):		
- Lymphocytes	25-40%	B lymphocytes: produce antibodies, antigen specific. T lymphocytes: antigen specific. - Cytotoxic T cells secrete substances to kill virus-infected and tumour cells. - Helper T cells activate and thus increase the production of antibodies by B lymphocytes. Can also activate macrophages. Natural killer cells (NK cells): kill virus-infected and tumour cells in similar way to cytotoxic T cells but do not need to recognise a specific antigen, i.e. are non-specific.
- Monocytes	2-8%	Leave blood to become macrophages. Engulf antigens and dead/dying cells by phagocytosis. Process and present antigens to T cells.
- Dendritic cells	~ 0.1%	Antigen presenting cells.

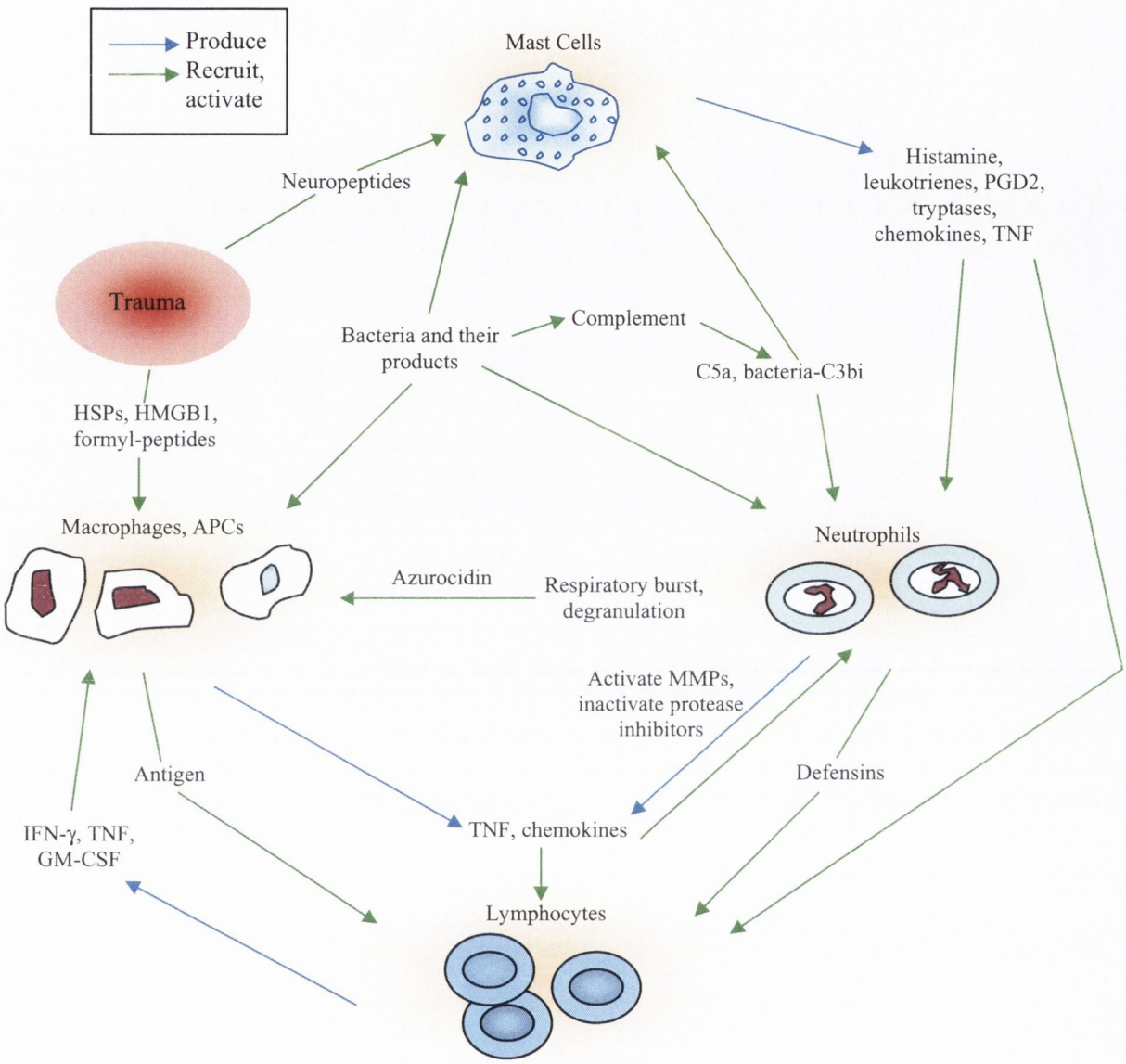

Figure 1.6 The early stages of inflammation following infection. Different cell types activate and recruit others in response to multiple inputs as a result of infection or trauma. Interaction among leukocytes, endothelium, platelets, and coagulation factors are not shown. Generation of stop signals is also not shown. Adapted from (Nathan, 2002).
APC = antigen presenting cell; PGD2 = prostaglandin D2; HSP = heat shock protein; HMGB1 = high mobility group 1; MMP = matrix metalloproteinase.

The first responders of the inflammatory response are mast cells and macrophages which are stationed in the tissue. Mast cells release factors such as histamines, eicosanoids, proteases, nitric oxide, kinins, cytokines, and chemokines in response to signals caused by tissue damage. Histamines and eicosanoids cause a vascular response involving vasodilation and vascular leakage. Mast cell proteases activate endothelial cells, causing the endothelium to become sticky to leukocytes. These vascular changes and increase in endothelium stickiness lead to the integral stage of inflammation where circulating leukocytes exit the circulation at the site of trauma in order to destroy invading pathogens and begin the process of tissue repair. In order for circulating leukocytes to be recruited to the site of inflammation, they start rolling onto the endothelial surface of the venules, adhere to the endothelium, and then transmigrate across the endothelial surface. These events are regulated by adhesion molecules expressed by both the endothelium and the leukocytes (Muller, 2002). The migration of leukocytes from the postcapillary venule is referred to as diapedesis or transmigration, and is thought to be stimulated by gradients of chemoattractants from the inflammatory environment.

Although the earliest cell type to the recruited to the site of inflammation is the neutrophil, macrophages are subsequently recruited and are responsible for various inflammatory, immunological, and tissue repair processes. Haematopoiesis, the development of mature blood cells, takes place in the bone marrow and lymphatic tissues. Like all blood cells, monocytes develop from pluripotential stem cells. During monocytopoiesis in adult bone marrow, these progress to monoblasts, promonocytes, and monocytes which then enter the blood stream. Finally, monocytes extravasate, either randomly or in response to inflammatory stimuli, and mature without proliferation into tissue macrophages (Friedman, 2002). A range of growth factors act together to control haematopoiesis, with relative combinations influencing the lineage specific differentiation. Development of pluripotent stem cells to myeloid progenitor cells is controlled by IL-3, and further differentiation to give rise to monocyte/macrophage destined cells is regulated by macrophage-colony stimulating factor (M-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) (Ihle, 1992; Becker *et al*, 1987). Both of these growth factors are subsequently necessary for blood monocyte survival and differentiation into macrophages (Becker *et al*, 1987). Macrophages derived from M-CSF-induced monocytes differ from those derived from GM-CSF-induced monocytes, with variations in their morphology, cell-surface antigens, and functions (Akagawa, 2002). IL-6 also plays a role in signalling differentiation towards mature phenotypes.

Macrophages are constitutively present in many tissues in the absence of inflammation, whereas monocytes are recruited to local sites following inflammatory stimuli. Mononuclear phagocytes adhere to cells such as lymphocytes, vascular endothelial cells, and to extracellular matrix proteins such as fibronectin and laminins by using integrins and other adhesion receptors on the monocyte cell surface. Adhesion is important in most monocyte/macrophage functions including antigen presentation, cytotoxicity, phagocytosis, chemotaxis, tissue recruitment, and production of inflammatory mediators (Pedraza *et al*, 2000). After entering a tissue, the monocyte develops into a tissue type macrophage which differs depending on the signals from the immediate surrounding tissue. For example, liver macrophages are known as Kupffer cells, interstitial or alveolar macrophages develop in the lung, osteoclasts are found in the bone, and microglial cells in brain tissue. Such a degree of heterogeneity allows flexibility in the functions of the macrophage as an important player in the innate immune response to inflammation and pathogenic invasion.

Under normal conditions tissue macrophages show little immunological activity and constitutive levels of cytokine production are low or non-existent. Upon inflammatory stimulation, macrophages are activated. Different subsets of T helper lymphocytes (T_H1 and T_H2) and their cytokine products act on macrophages to regulate their immunological properties. The T_H1 cytokine interferon gamma (IFN-γ) primes macrophages for enhanced cytotoxicity, increased production of pro-inflammatory cytokines, and enhanced MHC class II antigen expression. A further trigger such as the uptake of bacterial products induces macrophages to express their full biocidal activity. The macrophage itself regulates T cell IFN-γ expression with IL-12 whose production is stimulated by phagocytosis. T_H2 cells and monocytes produce IL-10, a deactivator of macrophage activation. Other T_H2 cytokines such as IL-4 and IL-13 also regulate the response by enhancing certain forms of macrophage immune activity and promoting humoral rather than cellular immunity. As well as their phagocytic activities, mononuclear phagocytes present antigens to MHC class II T lymphocytes and are essential sources of cytokines and chemokines.

1.4 REGULATION OF COAGULATION

Although activation of the coagulation system plays a crucial role in haemostasis and maintenance of vascular integrity, overproduction of fibrin can cause microthrombus formation at the site of inflammation leading to tissue ischaemia, and the spread of clot

formation from the site of injury leading to widespread thrombosis and disseminated intravascular coagulation (DIC). In addition to the activity of the fibrinolysis system in breaking down the fibrin clot, human plasma contains several factors that inhibit the coagulation system thus restricting and localising thrombosis and preventing vascular occlusion. The three major natural anticoagulant systems that regulate the coagulation system are the antithrombin (AT), tissue factor pathway inhibitor (TFPI), and activated protein C pathways.

1.4.1 The Antithrombin Pathway

Antithrombin (AT), a serine protease inhibitor (serpin) that is synthesised by the liver binds to and inactivates several proteases of the coagulation cascade. It acts as a major inhibitor of thrombin but also inhibits FIXa, FXa, FXIa, and FXIIa (Rosenberg, 1989). AT function is greatly enhanced by the formation of a complex with glycosaminoglycans, of which heparin is one. This causes a conformational change of the AT active site that is specific for thrombin and other coagulation factors (Rosenberg, 1989). Patients with congenital deficiency in AT or with variant AT lacking affinity for heparin are prone to recurrent venous thromboembolic events (Egeberg, 1965; Okajima *et al*, 1993).

The glycoprotein heparin cofactor II (HCII) also inhibits thrombin, but unlike AT it does not inhibit the activity of other coagulation factors (Tollefsen *et al*, 1982). As with AT, the action of HCII is enhanced by heparin, although the activity of the HCII-heparin complex is less than that of antithrombin-heparin complex. An association between lack of HCII and thrombosis has been reported (Sie *et al*, 1985), although the role played by HCII in regulating haemostasis has not been fully elucidated.

1.4.2 Tissue Factor Pathway Inhibitor

Tissue factor pathway inhibitor (TFPI), a protease inhibitor that circulates in the plasma mainly bound to lipoproteins, is predominantly produced by vascular endothelial cells. It has three tandem protease inhibitory domains (Wun *et al*, 1988) and is the main inhibitor of the FVIIa-TF complex which activates FX and FIX in the coagulation cascade. TFPI initially binds FXa, thus directly inhibiting it, and then inhibits FVIIa-TF activity by formation of a Xa-TFPI-FVIIa-TF complex (Broze and Miletich, 1987; Rao and Rapaport, 1987; Lindahl *et al*, 1992). TFPI contains a positively charged carboxyl terminus which is

thought to be responsible for the binding of TFPI to negatively charged glycosaminoglycans on the endothelium surface. Plasma levels of TFPI are rapidly increased after intravenous injection of heparin (Sandset *et al*, 1988; Lindahl *et al*, 1992). Heparin is thought to cause displacement of TFPI from the endothelial surface glycosaminoglycans and thus its release into the circulation, although evidence also points to release of TFPI by heparin from intracellular stores (Lupu *et al*, 1999). Patients with disseminated intravascular coagulation (DIC) may have decreased levels of TFPI (Bajaj *et al*, 1987).

1.4.3 The Protein C System

The Protein C pathway

Protein C (PC) is the vitamin K dependent zymogen of the serine protease activated protein C (APC). It has a molecular weight of approximately 62 kDa, a plasma concentration of 60-70 nM (3-5 μg/ml), and a half life of 6 to 10 hours (Esmon, 1987). First reported in 1976 as a new vitamin K-dependent protein, bovine protein C (Stenflo, 1976), PC is now known to play an important regulatory role in the coagulation, inflammation, and fibrinolysis pathways (Esmon, 2000a). In 1985, the protein C gene was sequenced (Foster *et al*, 1985). The gene, located on chromosome 2p13-14 (Patracchini *et al*, 1989), is approximately 11.2 kilobases in length and consists of nine exons and eight introns. The exons code for a 461 amino acid precursor protein which subsequently undergoes several post-translational modifications prior to secretion into the blood. The precursor protein is synthesised in the liver as a single chain of which 85-90% is converted into a two chain molecule made up of a light chain and a heavy chain linked by a connecting peptide. A signal peptide targets the protein to the endoplasmic reticulum before being cleaved from the protein. Nine Gla residues in the NH_2-terminal domain of the protein form the Gla domain which is a Ca^{2+} binding region that allows binding of PC to phospholipids in the cell membrane and to the endothelial protein C receptor (EPCR) (Oganesyan *et al*, 2002). Two epidermal growth factor (EGF)-like domains are thought to be important in achieving tertiary conformation and in binding to protein S. These are followed by the activation peptide, then by the serine protease catalytic domain. The activation peptide is released after cleavage by thrombin-thrombomodulin to give rise to APC. The domain structure of human protein C is shown in Figure 1.7.

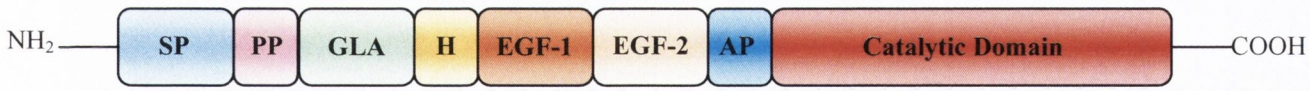

Figure 1.7 Domain structure of human protein C. SP = signal peptide; PP = propeptide; GLA = Gla domain; H = hydrophobic region; AP = activation peptide.

Protein C is physiologically activated by thrombin which cleaves the Arg169-Leu170 peptide bond releasing the twelve amino acid activation peptide from the PC heavy chain (Esmon, 1987) (Esmon, 1989) which is subsequently cleared from the circulation. This activation is increased at a rate of at least 1000 fold when thrombin is bound to thrombomodulin (TM), a thrombin receptor expressed predominantly on endothelial cells in the microvasculature (Esmon, 1989). Binding to TM blocks sites near the active centre of thrombin, thus hindering its ability to interact with fibrinogen and other procoagulants, and its ability to activate platelets and endothelium, whilst enhancing its activity towards PC. PC activation is also enhanced by approximately 20-fold when PC is bound to the endothelial protein C receptor (EPCR) (Stearns-Kurosawa *et al*, 1996).

APC circulates at a concentration of approximately 40pM and has a half life of 15 to 20 minutes before it is inactivated by several plasma serine protease inhibitors, including protein C inhibitor (PCI), α_1-antitrypsin (ATT), and α_2-macroglobulin (Gruber and Griffin, 1992; Heeb *et al*, 1989; Heeb and Griffin, 1988; Heeb *et al*, 1991). Once APC is generated, it binds to its co-factor, protein S (PS), and inactivates factors Va and VIIIa, both critical for thrombin generation, thereby attenuating the coagulation cascade. APC cleaves FVa in the prothrombinase complex and FVIIIa in the tenase complex at specific sites, resulting in the loss of interactions with FXa and prothrombin for FVa and with FIXa and FX for FVIIIa. FVIII is present as a complex with von Willebrand factor in plasma which protects it from proteolysis by APC, although once it is released as FVIIIa it is no longer protected. FVa is inactivated 5- to 10-fold faster than nonactivated FV. The rate of inactivation of FVa and FVIIIa by APC is accelerated by PS (Walker, 1980), an effect which requires the presence of phospholipid and Ca^{2+}. Protein S, a vitamin K dependent protein, circulates as a single chain glycoprotein with a molecular weight of 70 kDa. Approximately 30-50% of protein S is free in the circulation whilst the rest is complexed to C4b-binding protein (C4bBP) (Dahlback and Stenflo, 1981). Only free protein S possesses APC cofactor activity (Dahlback, 1986). The interaction with PS is thought to be through the EGF domains of PC. As well as its inhibition of coagulation, APC activity promotes

fibrinolysis, enhancing clot lysis, by neutralisation of PAI-1 activity (Esmon, 1989; Sakata *et al*, 1986). PCI inhibits APC activity by forming a 1:1 molar complex with APC, and the rate of this reaction is enhanced by heparin (Heeb *et al*, 1989). PCI also indirectly inhibits APC activity by inhibiting the thrombin-TM complex (Rezaie *et al*, 1995). ATT inhibits APC activity by disrupting its serine protease activity (Heeb and Griffin, 1988).

The protein C pathway functions primarily as a defence mechanism against thrombosis (Figure 1.8). The system acts in such a way that it can upregulate anticoagulant activity as the coagulation response increases, since as thrombin concentration rises increased amounts bind to TM, leading to more PC activation. Thrombin bound to TM is inactivated more rapidly than free thrombin by AT and PCI with a half life of less than 15 seconds (Esmon, 1987). Therefore once thrombin generation ceases, the protein C activation complex quickly stops generating APC. TM is located predominantly in the microvasculature which accounts for 99% of the total endothelial surface presented to blood, meaning that the concentration of membrane-bound TM in the microvasculature is 1000-fold higher than in the major vessels. Therefore, whilst thrombin is free in the major vessels, it is bound by TM as soon as it enters the microvasculature allowing for efficient local removal of thrombin from the circulation and rapid PC activation. This is consistent with the critical role played by the PC pathway in the control of microvascular thrombosis. Severe defects in the PC pathway are associated with microvascular thrombosis resulting in purpura fulminans which progresses rapidly unless treatment with PC supplementation is utilised (Dreyfus *et al*, 1991).

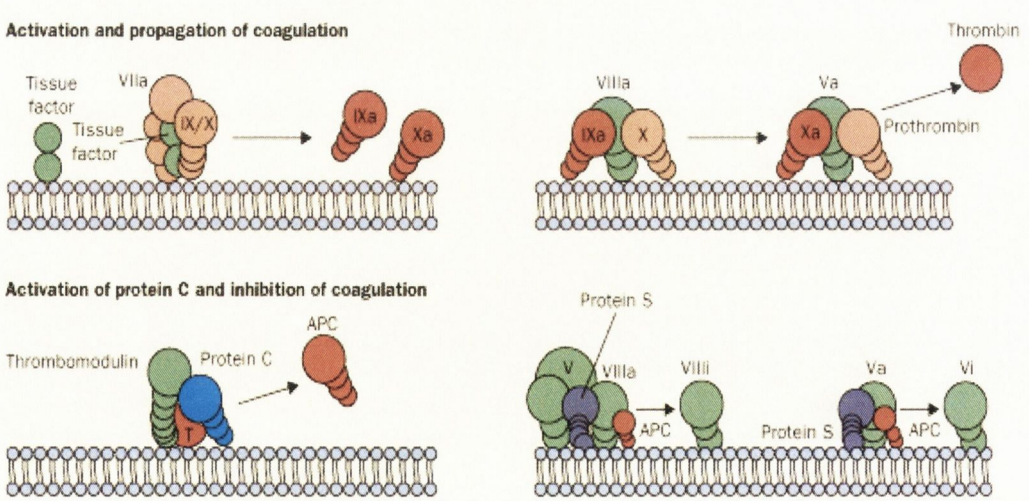

Figure 1.8 Schematic models illustrating some of the phospholipid-bound reactions that are involved in the activation and regulation of coagulation (Dählback, 2000).

The Endothelial Protein C Receptor

Both PC and APC bind with high affinity to human endothelial cells via a transmembrane protein known as the endothelial protein C receptor (EPCR) (Fukudome and Esmon, 1994). EPCR is a type I transmembrane protein that shares approximately 20% homology with the CD1/MHC class I family of proteins which are involved in the immune response (Fukudome and Esmon, 1994). APC and PC, *via* their Gla domains, both bind directly to EPCR on endothelial cells with similar affinity ($K_d \cong 30nM$) in a Ca^{2+} dependent fashion (Fukudome *et al*, 1996). This binding inhibits the anticoagulatory ability of APC to inactivate its natural substrate FVa (Regan *et al*, 1996) in a similar manner to the changes observed when thrombin complexes with its receptor thrombomodulin. PC binding to EPCR enhances the activation of PC by the thrombin-thrombomodulin complex as demonstrated by the 70-80% reduction in protein C activation with the use of blocking anti-EPCR antibodies (Stearns-Kurosawa *et al*, 1996).

The mature EPCR protein consists of 221 amino acids and has two extracellular domains which have homology to the α1 and α2 domains of CD1/MHC class I molecules. The third domain of CD1/MHC class I molecules, α3, is not present in the EPCR which instead has a transmembrane region followed by a short cytosolic tail (Villoutreix *et al*, 1999). The EPCR protein also contains four potential glycosylation sites. The α1 and α2 domains of CD1/MHC proteins form a groove which binds lipid antigens for presentation to T cells. The groove formed by the two domains in EPCR is relatively narrow and is lined with hydrophobic and polar residues with a few charged amino acids (Villoutreix *et al*, 1999). Mutagenesis studies have indicated that PC binding is mediated at a ten amino acid site at the tip of EPCR and does not involve the groove which binds a lipid molecule (Liaw *et al*, 2001). X-ray crystallography indicated a tightly bound phospholipid in the groove of EPCR whose presence is necessary for PC binding (Oganesyan *et al*, 2002).

The homology of EPCR to members of the CD1/MHC class I family (~20% sequence identity) can provide insights as to the function of EPCR, especially with regard to the role of such molecules in immunity and inflammation, mainly in antigen presentation and regulation of immunity. EPCR has even greater homology to the intracellular murine protein CCD41 (~62% identity) (Fukudome and Esmon, 1994) and the cDNAs encoding the two genes are identical indicating that the two proteins derive from the same gene (Rothbarth *et al*, 1999). Post-translational or post-transcriptional mechanisms are responsible for the functional difference between the two proteins. CCD41 is generally

associated with the centrosome and vesicles in the cell and is prevalent in the G2/M phase of the cell cycle whilst EPCR is directed to the cell surface. CCD41 is retained in the cytoplasm and subsequently directed to the centrosome as a result of the deletion of the signal peptide from the precursor protein. This was demonstrated by the exclusive targeting of fluorescently labelled CCD41 protein with intact signal peptide to the plasma membrane whilst CCD41 without the signal peptide is located in the centrosome (Rothbarth *et al*, 1999). The function of the murine CCD41 protein is currently unclear.

A soluble form of the EPCR (sEPCR) has also been reported (Kurosawa *et al*, 1997). Binding of PC to sEPCR does not result in enhanced APC generation by the thrombin-thrombomodulin complex and thus circulating levels of this soluble receptor may be important during a systemic inflammatory response (Kurosawa *et al*, 1997). Gu *et al* reported that endotoxin and thrombin elevate EPCR mRNA levels and EPCR shedding in rats, possibly increasing the thrombotic potential by diminishing APC activation and the ability of APC to inactivate FVa and FVIIIa (Gu *et al*, 2000). In normal individuals, the concentration of sEPCR (2.5 nM) is considerably less than the combined PC/APC concentration (65 nM) and would therefore be unlikely to have an effect on the anticoagulant function of the system. However, in severe sepsis the amount of PC can be less than 10% of normal levels (Smith and White, 1999) and sEPCR is elevated and therefore a significant amount of PC/APC is likely to be in complex and thus inactive (Liaw *et al*, 2000).

The importance of the EPCR in the host response to *E.coli* infection was recently demonstrated by blocking EPCR in baboons exposed to sub-lethal levels of *E. coli* thus causing a lethal response (Taylor *et al*, 2000). Animals receiving the EPCR-blocking antibody exhibited DIC, influx of neutrophils into the adrenal, renal, and hepatic microvasculature, increased thrombin-antithrombin complex, and increased levels of IL-6 and IL-8, leading to the conclusion that binding of PC/APC to the EPCR contributes significantly to the downregulation by APC of the inflammation and coagulation associated with *E. coli* infusion. Much research has focused on the interaction between APC/PC and endothelial cells. However, the level of PC activation via the thrombin-thrombomodulin complex is unlikely to explain the undoubted significance of PC in facilitating the anticoagulant and anti-inflammatory action of monocytes. Thus the recent discovery of the expression of the EPCR on the monocyte and neutrophil cell surface is an

important step in the further understanding of the modulatory effect of APC/PC on the activated monocyte (Galligan *et al*, 2001; Sturn *et al*, 2003).

Clinical Importance of Protein C

The importance of the protein C system as a host defence mechanism against excessive fibrin formation is highlighted by the severe thrombotic complications associated with homozygous protein C and protein S deficiency. The prevalence of inherited PC deficiency in the general population is estimated at 0.15-0.2% (Tait *et al*, 1995). Venous thrombotic disease is common later in life in patients with heterozygous PC deficiency or in homozygous patients that survive to adulthood (Allaart *et al*, 1993), although some heterozygous individuals are without symptoms. Inherited homozygous protein C deficiency results in neonatal purpura fulminans, which is characterised by disseminated intravascular coagulation and necrotic skin lesions (Branson *et al*, 1983; Seligsohn *et al*, 1984). The lethal neonatal thrombosis that usually occurs in these cases can be avoided by administration of APC (Dreyfus *et al*, 1991; Conard *et al*, 1993). This microvascular thrombosis, which is localised mainly in the skin, is similar to the lesions seen in meningococcaemia and other forms of septic shock. Mice with a targeted knockout of the thrombomodulin (TM) gene do not survive past 9 days as embryos, although heterozygous TM-deficient mice appear normal and are free of thrombotic complications despite a 50% reduction in TM levels compared to wild type mice (Healy *et al*, 1995). Protein S deficiency is also associated with increased risk of thrombosis (Faioni *et al*, 1997). Mutations in other genes involved in the protein C anticoagulant pathway have also been identified as risk factors for thrombophilia. These include mutations in the antithrombin gene, the thrombomodulin gene, the EPCR gene, and the factor V Leiden mutation which is associated with APC resistance (Koeleman *et al*, 1997).

1.5 SEPSIS AND SEPTIC SHOCK

Systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis, and septic shock describe an increasingly severe systemic response to infection. SIRS is defined as the presence of two or more objective signs of systemic inflammation. Patients with sepsis show evidence of infection and clinical manifestations of inflammation; severe sepsis involves the development of sepsis with at least one acute organ dysfunction; whilst septic shock patients develop persistent sepsis-induced hypotension, along with hypoperfusion abnormalities or organ dysfunction (Astiz and Rackow, 1998). The release of TNF and

other endogenous mediators leads to a range of pathophysiological responses such as fever, leukopenia, thrombocytopenia, haemodynamic changes, disseminated intravascular coagulation, and leukocyte infiltration and inflammation in various organs, all of which can ultimately lead to death. The most common cause of sepsis is bacterial infection (approximately 50-60% of cases), although fungal and viral infections can also be the cause. However, only 60% of sepsis cases are conclusively associated with a confirmed infection. The lungs, abdomen, and urinary tract are the most frequent sites of infection (Astiz and Rackow, 1998). It is estimated that over 750 000 cases of severe sepsis occur in the United States each year and septic shock develops in approximately half of these (Angus *et al*, 2001). The mortality rate, which increases with age, has been reported to range from 7% and 16% in SIRS and sepsis respectively, to 20% and 46% in severe sepsis and septic shock (Rangel-Frausto *et al*, 1995), although estimates of mortality vary slightly in different studies.

Sepsis and septic shock demonstrate the intricate relationship that exists between the inflammation and coagulation pathways. An initial toxic stimulus, such as that caused by LPS, triggers production of macrophage derived proinflammatory cytokines (mainly TNF-α, IL-1) and monocyte adherence to endothelial cells. Adhesion molecules are expressed on endothelial cells and neutrophils (Muller, 2002), and the transmigration of neutrophils into the extravascular space results in microvascular and tissue injury. All activated cells release secondary inflammatory mediators, including cytokines such as IL-6 and IL-8, leukotrienes, PAF, prostaglandins, and complement. Nitric oxide is also produced, and this is believed to play an important role in the vasodilation and hypotension seen in septic shock. Endothelial cells can cause activation of platelets and increased production of procoagulants, triggering microthrombosis. Endotoxin, TNF-α, and IL-1 increase TF expression on endothelial cells and macrophages, leading to initiation of the tissue factor-dependent coagulation pathway (van der Poll *et al*, 1990), increased fibrin deposition, and eventually disseminated intravascular coagulation (DIC) can occur, causing life-threatening tissue ischaemia. This involves a low oxygen state due to inadequate blood flow leading to hypoxia in the tissue. The activation of coagulation is amplified by impaired function of the protein C-protein S inhibitory pathway and decreased AT levels (Levi *et al*, 1993; Lorente *et al*, 1993). In sepsis, as well as increased inflammatory and coagulant activity, normal fibrinolysis is suppressed by elevation of levels of PAI-1 and thrombin activatable fibrinolysis inhibitor (TAFI), both naturally occurring inhibitors of fibrinolysis (Lorente *et al*, 1993) further promoting the procoagulant state. Vessel dilation

caused by free radicals, histamine, prostaglandins, prostacyclin, and the kinin and tachykinin family of molecules, combined with the effects of cytokines on endothelial cells, contribute to increased vascular permeability for fluids and low-molecular weight substances, causing localised accumulation of excessive fluid in the subcutaneous tissues (oedema). If the process is widespread, a capillary leak syndrome may result. Erythrocytes may also leak out, causing bleeding. Anti-inflammatory substances such as IL-4, IL-10, IL-1Ra are also produced during septic shock, leading to a period of immune depression following the initial inflammatory response. However, these mediators appear to be ineffective in controlling the organ dysfunction, tissue damage, and thrombosis seen in septic shock.

The overall view of sepsis can be seen as a cycle of increased inflammation and coagulation as a result of the loss of homeostasis between the inflammation, coagulation, and fibrinolysis pathways (Healy, 2002). A feedback amplification loop is initiated by the systemic inflammatory response resulting in tissue injury and induced expression of TF on monocytic and endothelial cell surfaces. This leads to the activation of coagulation through the tissue factor-dependent pathway, accelerating the production of thrombin. The endothelial injury exacerbates inflammation by increasing neutrophil activation, increasing neutrophil-endothelial adhesion, and causing additional release of pro-inflammatory cytokines. These processes further increase endothelial dysfunction and weaken microvascular function, thus decreasing tissue perfusion, leading to ischaemia and acute organ failure. During this process, naturally occurring modulators of homeostasis such as PC and AT are depleted and become deficient as the body attempts to return to a normal state. This cycle of inflammation and coagulation fuels the progression of the disease resulting in organ failure and eventually death in a large number of patients.

1.5.1 The Protein C Pathway in Sepsis

A pro-coagulant response occurs in sepsis, and this activity is thought to cause the high level of morbidity observed in the disease, survivors of which often suffer from mutilating complications such as amputations and skin grafts. DIC and an acquired deficiency of natural anticoagulants, including protein C, protein S, and antithrombin are seen in most patients with the disease (Heyderman, 1993). However, plasma protein C concentrations are reduced to a greater extent than the other two anticoagulants (Fijnvandraat *et al*, 1995), and decreased levels of APC are associated with a greater risk of death (Heyderman, 1993;

Powars *et al*, 1993; Fijnvandraat *et al*, 1995). The conversion of protein C to APC depends on the presence of thrombin coupled to thrombomodulin (Esmon, 1989). This conversion may be impaired during sepsis due to down-regulation of thrombomodulin by inflammatory cytokines (Conway and Rosenberg, 1988) possibly accounting for the decreased levels of APC seen in patients. There is a strong correlation between the severity of protein C deficiency, the extent of thrombosis, and adverse clinical outcome in sepsis (Powars *et al*, 1993; Fijnvandraat *et al*, 1995), pointing to a vital role played by APC in the pathophysiology of the disease.

1.5.2 Treatment of Sepsis

The conventional strategy for sepsis treatment often involves a range of therapies to deal with the effects of uncontrolled inflammation and coagulation. The underlying infection must be treated, usually with antibiotics, or the site of infection erradicated by surgical procedures or invasive radiology. Supportive care is used to stabilise organ systems and limit tissue damage. This involves the maintenance of adequate arterial oxygen saturation, colloid fluid resuscitation, and correction of acid-base electrolyte abnormalities (Astiz and Rackow, 1998). Other treatments include administration of low dose steroids and the maintenance of normoglycaemia (Chadda and Annane, 2002; Van den Berghe, 2002).

Efforts to develop more effective therapies against sepsis, mostly involving the use of agents to block host cell activation or inflammatory mediators, have generally failed. Randomised trials of a wide range of agents including anti-endotoxin antibody, anti-TNF antibody, soluble TNF receptor, IL-1 receptor antagonist, anti-bradykinin, PAF antagonist, steroids, non-steroidal anti-inflammatory drugs, and prostaglandin inhibitors were unsuccessful in improving survival from sepsis and septic shock (Astiz and Rackow, 1998). The failure of such therapies could be due to the complexity of septic shock. Rather than viewing inflammation as the dominant force in sepsis, the current understanding that the disease is caused by a loss of homeostasis between inflammation, coagulation, and fibrinolysis, could explain the failure of specific anti-inflammatory therapies. Additionally, the use of agents that only down-regulate the immune response may have implications for subsequent infectious complications and despite decreasing inflammation may adversely affect the clearance of the primary infection.

Endogenous APC is an important inhibitor of the coagulation pathway as it acts to inhibit FVa and FVIIIa, factors involved in rate-limiting steps of the cascade (Esmon, 1989). Additionally, APC acts to promote fibrinolysis by neutralising the inhibitors PAI-1 and TAFI and accelerating t-PA dependent clot lysis (Grinnell and Joyce, 2001). APC also appears to limit the amplification cycle of inflammation and coagulation seen in sepsis through its anti-inflammatory activity (Grey *et al*, 1994; White *et al*, 2000b; Yuksel *et al*, 2002). APC therefore has the potential to restore the balance in the three major processes driving sepsis and organ dysfunction: increased coagulation, suppressed fibrinolysis, and upregulated inflammation (Esmon *et al*, 1991; Healy, 2002) (Figure 1.9). The initial evidence for a therapeutic role of APC in sepsis came from Taylor *et al* who showed that APC administration prevents the coagulopathic, hepatotoxic, and lethal effects of *Escherichia coli* infusion in the baboon (Taylor *et al*, 1987). APC, but not active site blocked APC, was subsequently shown to reduce LPS-induced pulmonary vascular injury and LPS-induced pulmonary accumulation of leukocytes in rats (Murakami *et al*, 1996). Other studies have found APC treatment to inhibit LPS-induced pro-inflammatory cytokine production *in vitro* thereby potentially minimising leukocyte activation and endothelial injury in the disease (Grey *et al*, 1994; White *et al*, 2000b; Yuksel *et al*, 2002). Blocking of the EPCR in baboons exposed to sub-lethal levels of *E. coli* causes a lethal response (Taylor *et al*, 2000). Finally, clinical trials of PC or APC treatment in meningococcaemia or other forms of sepsis have provided the most compelling evidence for the potential of APC/PC as a therapeutic agent in sepsis. A series of clinical studies suggested a trend towards improved survival of septic patients treated with PC (Smith *et al*, 1997; Hartman *et al*, 1998; Smith and White, 1999; White *et al*, 2000a), and these positive results were confirmed in a large-scale clinical trial. In the first randomised phase III trial to use recombinant APC in severe sepsis patients (PROWESS), a total of 850 patients with systemic inflammation and organ failure were treated with recombinant human APC (rhAPC), with 840 patients in the placebo group. Treatment with rhAPC was associated with a reduction in the relative risk of death of 19.4% (95% CI 6.6-30.5), and an absolute reduction in the risk of death of 6.1% ($p=0.005$) (Bernard *et al*, 2001).

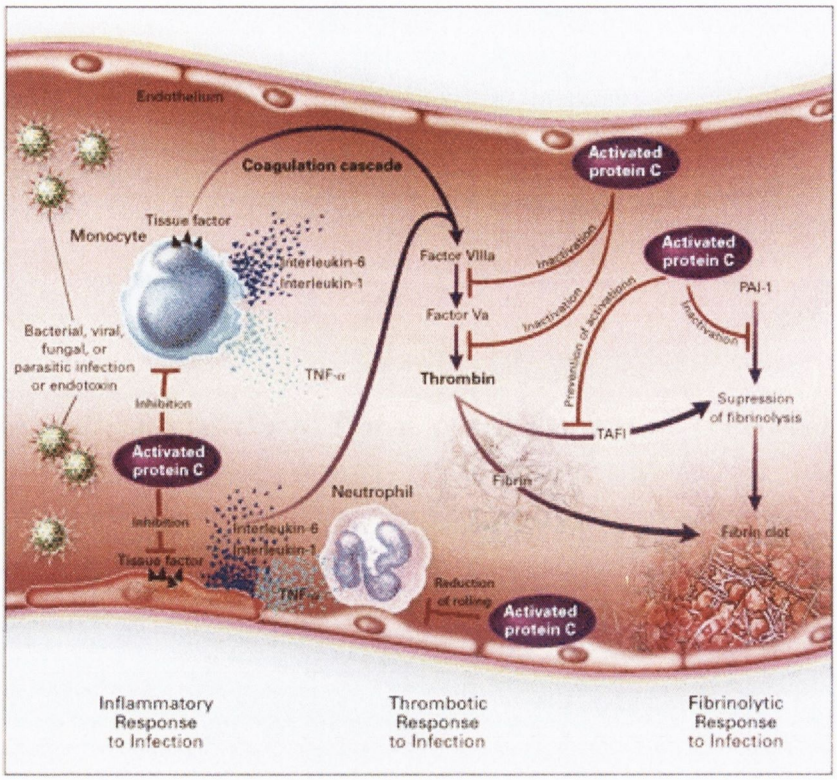

Figure 1.9 Potential actions of APC in modulating the systemic inflammatory, procoagulant, and fibrinolytic host responses to infection. Pro-inflammatory cytokines stimulate coagulation by inducing the production of tissue factor from monocyte and endothelial cells leading to the production of thrombin and the formation of a fibrin clot. Inflammatory cytokines and thrombin can impair the fibrinolytic response by stimulating the release of PAI-1 from platelets and the endothelium. Thrombin is additionally capable of stimulating multiple pathways of inflammation and further suppressing fibrinolysis by activating TAFI. APC can intervene at multiple points during the systemic response to infection. It exerts an antithrombotic effect by inactivating FVa and FVIIIa, and thus limiting thrombin generation. This decrease in thrombin reduces the inflammatory, procoagulant, and antifibrinolytic responses induced by thrombin. APC also inhibits the production of inflammatory cytokines by monocytes and indirectly increases fibrinolysis by inhibiting PAI-1. Reprinted from (Bernard *et al*, 2001).

1.6 THE TOLL-LIKE RECEPTOR FAMILY

1.6.1 Toll-like Receptors

There have been considerable advances in recent years in the understanding of how the innate immune system is capable of polarising its response to a particular stimulus. It was hypothesised that cells of the innate immune system express protein receptors that recognise conserved molecules from pathogenic organisms (Janeway, 1989). These receptors, known as pattern recognition receptors (PRRs), recognise endogenous signals from damaged or stressed tissues or pathogen associated molecular patterns (PAMPs) such as LPS, peptidoglycan (PGN), and lipoteichoic acids (LTAs), and alert the immune system to abnormal cell damage and death, or to the presence of pathogens (Gallucci and

Matzinger, 2001). A number of PRR families have been identified, including scavenger receptors, mannose receptors, CD14, and Toll-like receptors (TLRs) (Medzhitov, 2001; Peiser *et al*, 2002).

TLRs are a family of transmembrane receptors which share a conserved sequence in the intracellular cytosolic portion of the protein known as the Toll/IL-1 receptor (TIR) domain. This domain, a protein-protein interaction molecule which is found conserved in several transmembrane proteins in both plants and animals, initiates the signaling cascade that is triggered by recognition of conserved molecular patterns common to classes of pathogens, otherwise known as PAMPs, or by endogenous damage signals generated at sites of inflammation. TLRs are also characterised by an extracellular leucine-rich repeat (LRR) domain. LRRs are found in a variety of proteins, where they are involved in ligand recognition and signal transduction. The LRR domain and the TIR domain are linked by a single transmembrane crossing. TLRs, expressed widely by cells of the immune system and by many other cell types, detect a diverse array of pathogens including bacteria, mycoplasma, fungi, viruses, and protozoa. Monocytes/macrophages express mRNA for most TLRs except TLR3 (Takeda *et al*, 2003). Expression of TLRs in dendritic cells differs among their subsets, while mast cells express TLR2, 4, 6, and 8 but not TLR5. Phagocytes show abundant expression of all known TLRs although several are preferentially expressed in B cells (Takeda *et al*, 2003). In addition to expression in innate immune cells, TLRs are expressed in other cells that play a role in the inflammatory response, such as the epithelial cells of the mucosal surfaces of the respiratory and intestinal tract and endothelial cells of the microvasculature. Most tissues express at least one TLR, for example, TLR2, 4, and 6 have been identified in heart tissue (Knuefermann *et al*, 2002).

The first member of the Toll family to be identified was the *Drosophila melanogaster* Toll. This was identified as a part of the pathway controlling dorsoventral patterning in embryos (Hashimoto *et al*, 1988), but was later discovered to also control anti-fungal immunity in adult fruit flies (Lemaitre *et al*, 1996). In humans, TLR4 was the first TLR to be characterised (Medzhitov *et al*, 1997). TLR4 is discussed in more detail in Section 4.1.1. The discovery of TLR4 lead to an explosion of research in this area. To date, ten known TLRs have been identified in mammals (Table 1.3), each with a distinct function in innate immune recognition. Dozens of ligands been have also been detected. More TLRs and more ligands will undoubtedly be discovered in the future. Ligands so far identified are

diverse in both structure and in origin, although most are conserved microbial products (PAMPs). Although some TLRs seem to be specific with regard to their stimuli, others are capable of recognising several structurally diverse ligands, probably made possible by their interaction with different combinations of accessory proteins.

Table 1.3 Mammalian Toll-like receptors and their ligands.

TLR	Ligand (origin)	Associated Proteins
TLR1	Tri-acyl lipopeptides (bacteria, mycobacteria)	Signalling only as dimer with TLR2
	Soluble factors (*Neisseria meningitides*)	
TLR2	Lipoproteins/lipopeptide (variety pathogens)	CD11a/CD18, CD11b/CD18, CD14,
	Peptidoglycans (Gram positive bacteria)	TLR1, TLR6, dectin-1
	Lipoteichoic acid (Gram positive bacteria)	
	Mannuronic Acids	
	Bacterial Fimbriae	
	CMV virions	
	Yeast cell walls	
	Porins (*Neisseria*)	
	Phenol soluble modulin	
	Some atypical LPS	
	HSP70 (host)	
TLR3	Double stranded RNA (virus)	
TLR4	LPS (Gram negative bacteria)	LBP, CD14, MD-2, CD11b/CD18
	HSP60 (host & bacterial)	
	HSP70 (host)	
	Taxol (plant)	
	Mannuronic acid polymers	
	Flavolipins	
	Teichuronic acids	
	Bacterial fimbriae	
	Coat protein (RSV)	
	Envelope proteins (MMTV)	
	Fibrinogen (host)	
TLR5	Flagellin	
TLR6	Di-acyl lipopeptides (mycoplasma)	Forms dimer with TLR2
	Phenol-soluble modulin	
TLR7	Imidazoquinoline anti-virals (synthetic compounds)	
	Loxoribine (synthetic compounds)	
	Bropirimine (synthetic compounds)	
TLR8	Imidazoquinoline anti-virals (synthetic compounds)	
TLR9	Unmethylated CpG DNA motifs (bacterial DNA)	
TLR10	Unknown	

1.6.2 TLR Signalling Pathways

The signalling pathways of TLRs are generally shared, although differences are emerging which point to some specificity. The activation of diverse TLRs can result in different consequences within a cell as each TLR induces a common set of genes but can also induce a number of genes specific to that TLR, presumably to allow the cell to respond appropriately to different pathogenic stimuli. TLRs activate a common signalling pathway mediated through the TIR domain which culminates in NF-κB and mitogen-activated protein kinase (MAP kinase) (such as p38 and JNK) activation leading to the activation of a variety of genes including many involved in inflammation and host defense. Signalling is initiated by the recruitment of the adaptor protein MyD88 via TIR-mediated interactions with the TLR (Medzhitov *et al*, 1998). Studies of MyD88-deficient mice have revealed that this protein, which has a TIR domain in its C-terminal portion and a death domain in its N-terminal portion, plays a critical role in the response to LPS and IL-1 (Kawai *et al*, 1999). MyD88 recruits IL-1R-associated kinase (IRAK), a death domain containing serine/threonine kinase (Muzio *et al*, 1997) which is activated by phosphorylation to associate with TNFR-associated factor 6 (TRAF6). The IRAK family has four members, IRAK-1, IRAK-2, IRAK-M, IRAK-4, all of which contain a death domain and a kinase domain. IRAK-4 is important in the TLR4 dependent signalling pathway. Like MyD88, TRAF-6 deficient mice exhibit impaired responses to LPS and IL-1 indicating that it too is essential for TLR signalling downstream of MyD88 (Lomaga *et al*, 1999). Signalling via the MyD88-dependent pathway, through TLR, MyD88, IRAK, and TRAF6 leads to stimulation of the IκB kinase (IKK) complex resulting in NF-κB induction. Finally, an adaptor molecule known as Toll-interacting protein (Tollip) thought to negatively regulate the TLR-mediated signalling pathway has also been identified. Overexpression of Tollip blocks NF-κB activation in response to TLR2 and TLR4 agonists (Zhang and Ghosh, 2002).

Other TLR signalling pathways, such as the MyD88-independent LPS response, have been described. LPS can induce NF-κB and JNK activation in MyD88-deficient mice, although the response is slower than that achieved through the MyD88-dependent pathway (Kawai *et al*, 1999). A recently identified molecule named TIR domain-containing adaptor protein (TIRAP)/MyD88-adaptor-like (MAL) might be involved in this MyD88-independent LPS-induced activation of NF-κB mediated by TLR4 (Horng *et al*, 2001; Fitzgerald *et al*, 2001).

Like TLR4, other TLRs also appear to have their own signalling pathways in addition to the common MyD88-dependent pathway (Takeda *et al*, 2003).

Nuclear Factor-kappa B (NF-κB)

NF-κB is the name for a group of inducible transcription factors whose nuclear translocation from the cytosol is responsible for the rapid expression of mediators involved in several processes including inflammation, coagulation, and the response to stress (Barnes and Karin, 1997). Several signalling pathways lead to NF-κB activation, including cellular activation by microbial ligands via members of the TLR family. NF-κB functions as a heterodimer, consisting of subunits p65 and p50 (Siebenlist *et al*, 1994). In unstimulated cells a family of inhibitory proteins known as IκBs bind NF-κB sequestering it in the cytoplasm and preventing nuclear translocation, and thus activation of target genes. (Baldwin, 1996). Factors such as LPS, viruses, oxidants, and protein kinase C activators cause IκB phosphorylation, ubiquitination, and eventual proteolytic degradation, which releases NF-κB allowing it to be translocated to the nucleus where it binds to specific sequences in the promoter region of a range of target genes (DiDonato *et al*, 1996). IκB kinase (IKK), responsible for IκB phosphorylation, is made up of two subunits, IKKα and IKKβ. Increased nuclear translocation of NF-κB, and thus pro-inflammatory activity, has been implicated in a range of disease states, including sepsis, malignancy, and chronic inflammatory disorders (Barnes and Karin, 1997).

Other Transcription Factors Activated in the TLR-Mediated Signalling Pathway

The activating protein-1 (AP-1) family of transcription factors consists of homodimers and heterodimers of the Jun and Fos family (Karin *et al*, 1997). As well as forming dimers within the AP-1 family, Jun proteins also form heterodimers with members of the cAMP response element binding (CREB)/activating transcription factor (ATF) family of transcription factors. This enables them to bind to the cAMP response element (CRE) as well as the 12-O-tetradecanoylphorbol-13-acetate response element which AP-1 transcription factors bind to. AP-1 is upregulated through phosphorylation of JNK and extracellular-signal regulated kinase (ERK) MAP kinases, stimulated by LPS and peptidoglycan. Nuclear factor for IL-6 expression (NF-IL6), a member of the CCAAT/enhancer binding protein (C/EBP) family, is activated in response to inflammatory stimuli and plays a role in the macrophage response to inflammation, especially in the response to LPS (Poli, 1999). Members of the interferon regulatory factor (IRF) family of transcription factors are important regulators of the cellular response in

host defence (Taniguchi *et al*, 2001). IRF-3 is thought to be involved in the MyD88-independent LPS signalling pathway.

1.7 SINGLE NUCLEOTIDE POLYMORPHISMS

With recent advances in the Human Genome Project and improved methods for discovering DNA sequence variation, the identification of genes contributing to the susceptibility or resistance to many human diseases, or to response to medication, is becoming increasingly achievable. Many genes for human diseases have been identified by genome-wide linkage studies and positional cloning. Over 10000 human diseases caused by single gene defects have been described. Examples include cystic fibrosis, alpha-1-antitrypsin deficiency, sickle cell disease, thalassaemia, haemochromatosis, haemophilia, Huntingdon's disease, muscular dystrophy, glucocorticoid-remediable aldosteronism (GRA), Liddle's Syndrome and other hypertensive conditions, as well as cardiac myopathies, dysplasias, and arrhythmias that result in Sudden Cardiac Death. Although individuals have single gene defects, there can be considerable variation in the progression, complications and outcome of the disease. This may be due to the interaction of the disease gene with other genes, impacting on the final disease presentation.

The diseases listed above are conditions where a mutation in a single gene is enough to cause the disease. It is significantly more difficult to identify genes that contribute to diseases whose phenotypes are affected by multiple genes, each with a small contribution to the disease aetiology, and for which environmental factors are also important. Examples of such polygenic diseases are diabetes, coronary heart disease, hypertension, epilepsy, asthma, and certain psychiatric disorders. These diseases have a low heritability compared to monogenic diseases, indicating that no single genetic factor is responsible for causing the disease. It is thought that the incidence of any complex disease is dependent on a balance of risks. There is a balance between gene variants (alleles) with positive and negative effects, and between environmental factors with positive and negative effects. Too many negative factors, both genetic and environmental, can tip the balance towards disease. Large-scale studies can help to isolate genes that contribute to complex diseases since individuals with alleles that predispose them towards a disease will statistically be more likely to be affected. Similarly, those with alleles that protect them from a disease will statistically be less likely to be affected.

It is thought that approximately 80-90% of sequence variation in human DNA is caused by differences in single bases, called single nucleotide polymorphisms (SNPs) (Collins *et al*, 1998), the rest being attributable to insertions or deletions of one or more bases, repeat length polymorphisms, and rearrangements. Those SNPs that are common in different ethnic groups are thought to have arisen in the early stages of human evolution. This small number of common variants make up the bulk of heterozygosity in the human population, whilst other newer variant sites are rare. These common variants have therefore been the focus of many association studies, since they are at a sufficiently high frequency in control and patient populations for this type of study. An association study involves the comparison of a large cohort of patients who have a particular disease with a control group in order to look for factors which are found significantly more or less frequently in the patients than in the control population. Approximately 1.4 million SNPs have so far been identified and mapped, with an average density of one SNP for every 1.9 kilobases (Sachidanandam *et al*, 2001). Approximately 60 000 SNPs are estimated to fall within exons, and 85% of exons are within 5 kb of the nearest SNP (Sachidanandam *et al*, 2001). Although most SNPs do not affect gene function, those located in the coding (exons) or in the regulatory region of genes are more likely to have functional effects than SNPs in other areas.

Genetic diversity is not only limited to single polymorphisms, but also occurs at the level of specific combinations of alleles, called haplotypes, observed at closely linked sites. Because of linkage disequilibrium, certain alleles of genetic markers, i.e. certain haplotypes, located close to a mutation that causes a disease are often found associated with the disease. It is possible to define common haplotypes using a set of polymorphic genetic markers, and to study each haplotype for association with disease. Once the haplotype associated with a disease is located, the region can then be further analysed to reveal the disease-associated mutation. The advantage of this method is that regions of the genome can be studied for disease association without the need for the discovery of a functional variant. The density of markers required and the area of genome needed to be covered by the markers for such a study is not yet clearly defined. However, since SNPs occur on average every 1 to 2 kilobases, they would be suited to haplotype analysis.

The HLA locus on chromosome 6 is an area of high heterozygosity. This area is of importance and interest in the field of immunology. It is thought that the high nucleotide diversity in this area is due to balancing selection (Horton *et al*, 1998). On the other hand,

regions of low diversity are seen in particular on the sex chromosomes (Sachidanandam *et al*, 2001).

Since November 2000, a map of SNPs has been publicly available. This is integrated with the genetic and physical maps of the human genome sequence. It is anticipated that this SNP map will be used in the application of human population genetic studies, disease candidate gene studies, and, in the future, for genome-wide association scans. The map will also facilitate the study of haplotype diversity in the human genome. Finally, where a candidate disease gene has been identified by linkage to chromosomal position, biological function, or expression pattern, it will be possible to then study any association between allelic variation and disease (Sachidanandam *et al*, 2001). It is hoped that such studies will affect the diagnosis, treatment, and understanding of common human diseases, with the ultimate aim of developing new, more effective therapies. However, it is important to note that for complex diseases, compared to diseases with one wild type and one major susceptibility allele, more markers, larger study sizes, more studies, and more powerful statistical tools are needed than are used in most current studies in order to reduce the risk of obtaining false positive results.

CHAPTER 2

Materials and Methods

2.1 MATERIALS

2.1.1 Reagents

All chemical reagents were supplied by Sigma (St Louis, MO, USA) or Gibco/Invitrogen (Carlsbad, CA, USA) unless otherwise stated.

2.1.2 Solutions

RNA solutions were made up with DEPC H_2O (0.1% v/v diethylpyrocarbonate in deionised H_2O, shaken, allowed to stand 3-6 hours or overnight, and autoclaved prior to use).

2.1.3 Oligonucleotide Synthesis

Lyophilised oligonucleotides were supplied by Sigma Genosys (The Woodlands, TX, USA) and reconstituted with nuclease free H_2O to a stock concentration of 100 μM.

2.2 METHODS

2.2.1 Cell Culture Protocols

Subculture of THP-1 cells

The THP-1 human monocyte suspension cell-line (American Tissue Culture Collection, Virginia, USA) was initially cultured in culture medium consisting of RPMI 1640, supplemented with 15% foetal calf serum (FCS), L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 μg/ml) (all Gibco/Invitrogen), and 10 mM HEPES (Sigma) in 50 ml cell culture flasks containing gas exchange filters (NUNC Brand Products, Roskilde, Denmark). After 72 to 96 hours incubation at 37°C in 5% CO_2, the cells were centrifuged at 55 x g for 5 minutes, the medium was discarded, and the cells were resuspended in growth medium (RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin). The subculture routine employed in order to maintain a cell concentration between 2 x 10^5 and 1 x 10^6 viable cells/ml entailed the addition of fresh culture medium every 72 to 96 hours, with incubation at 37°C in 5% CO_2

in 200 ml cell culture flasks containing gas exchange filters (Iwaki, Tokyo, Japan). All reagents used for tissue culture were certified pyrogen-free. All tissue culture was performed in a laminair hood in sterile conditions.

Long term storage of cell lines

Frozen stocks of the THP-1 cell line were prepared as soon as possible after receipt in order to ensure low passage number and reduced risk of contamination. Cells were counted, pelleted by centrifugation for 5 minutes at 55 x g, and resuspended in cryoprotectant medium consisting of 50% FCS, 45% RPMI 1640, and 5% DMSO (Sigma) at a concentration of 5-10 x 10^6 cells/ml. Aliquots of 1 ml were stored at –70°C in 2 ml screw cap tubes (Sarstedt, Numbrecht, Germany) until required for further use. For long term storage, stocks were stored in liquid nitrogen.

Growing up frozen cell line stocks

Frozen aliquots of THP-1 cells were allowed to defrost at 37°C until barely defrosted. Cells were then added to 10 ml of culture medium (pre-incubated at 37°C) consisting of growth medium containing an additional 5% FCS. Cells were pelleted for 5 minutes at 55 x g, the supernatant discarded, and resuspended in 10 ml of culture medium (as above) and incubated at 37°C in 5% CO_2 in 50 ml tissue culture flasks (NUNC Brand Products). After 72 to 96 hours incubation, the regular subculture routine (see above) was employed for maintainence of the correct cell concentration and conditions.

Haematocytometer cell counting and viability

Cells were counted by trypan blue staining and the use of a haemocytometer and light microscope. Cell viability was determined using the trypan blue exclusion test.

2.2.2 Primary Human Monocyte Isolation

Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Fresh PBMCs were obtained from healthy donors using the Lymphoprep density gradient centrifugation method (Nycomed, Oslo, Norway). All procedures took place on ice and in a category 2 biohazard laminair hood. Briefly, 250 to 300 ml venous blood (+10% sodium citrate) was collected from each donor, 150 ml of which was diluted in an equal amount of phosphate buffered saline (PBS) and slowly layered over Lymphoprep (2:1 ratio of blood to Lymphoprep), in 50 ml tubes (Sarstedt). Samples were then centrifuged at 400 x g for 40

minutes in a swing out rotor with no brake applied. PBMCs and platelets collected on top of the gradient due to their lower density, whereas red blood cells, which have a higher density, pelleted below the gradient. The mononuclear layer was removed with a pasteur pipette, and the lymphoprep step was repeated to ensure the removal of all red blood cells. The PBMCs were then washed twice with PBS for 10 minutes at 400 x g (no brake), removing any remaining lymphoprep and platelets. Viable cells were counted by means of a haemocytometer, with trypan blue exclusion. PBMCs were kept on ice for further cell separation.

Magnetic Separation of Monocytes

MACS (monoclonal antibody cell sorter) microbeads (Miltenyi Biotec, Bergish Gladbach, Germany) conjugated to antibodies directed against CD3 (T cells), CD7 (dendritic cells), CD19 (B cells), CD45RA (B cells, NK cells, naive T cells), CD56 (natural killer cells), IgE (basophils), and CD 61 (expressed on thrombocytes, megakaryocytes, osteoclasts, and endothelial vessel cells) were used to deplete the non-monocyte fraction of the PBMCs, and thus allow for the negative selection of monocytes and macrophages. A varioMACS magnet (Miltenyi Biotec) was used, allowing the separation of up to $2x10^9$ cells.

Whole blood (100ml) was centrifuged at 2000 x g for 20 minutes (no brake applied). The plasma was removed and filtered to obtain autologous platelet free plasma. Pelleted PBMCs were resuspended in 60 µl of buffer (PBS containing 4% autologous serum and 250 µM EDTA) for every 10^7 total cells, and a small aliquot was retained for FACS analysis. For every 10^7 total cells, 20 µl of hapten-antibody cocktail (to label CD3, CD7, CD19, CD45RA, CD56, and IgE positive cells) and 20 µl of autologous serum was added to the PBMCs in buffer. Following a 5 minute incubation at 6-12°C, cells were washed twice with 10-20X labelling volume of buffer, and resuspended in 60 µl buffer, 20 µl autologous serum, 20 µl anti-hapten microbeads, and 10 µl anti-CD61 microbeads per 10^7 total cells. After a 15 minute incubation at 6-12°C, cells were washed twice with 10-20X labelling volume of buffer. The cell pellet was diluted in 500 µl buffer for 10^8 total cells. A type LS^+ separation column was placed in the varioMACS magnet and washed with 3 ml of buffer. The magnetically labelled cells were applied to the column and washed through with 4 x 3ml buffer. The eluent, containing the negative monocyte fraction was centrifuged, diluted in 1ml of buffer, and viable cells counted by means of a haemocytometer, with trypan blue exclusion. Monocytes were then diluted to a concentration of $1x10^6$ cells/ml in RPMI containing 10% autolgous serum. The positive

fraction, left behind in the LS^+ column was flushed into a second collecting tube away from the magnet. This fraction, as well as small portion of the negative fraction (monocytes), were kept for FACS analysis in order to monitor the cell purity of the monocytes isolated.

2.2.3 Flow Cytometry

Purity of Isolated Cells

Purity of the isolated monocytes was determined by fluorescent-activated cell sorting (FACS) analysis. After one wash step in buffer (PBS with 1% BSA and 0.15% sodium azide, 0.2 µm filtered), a minimum of 10 000 cells were resuspended in 100 µl of buffer and incubated with r-phycoerythrin (RPE)-conjugated mouse anti-human CD14-specific monoclonal antibody (1:100 dilution) (Dako, Glostrup, Denmark) for 30 minutes at 4°C in the dark. Cells were washed twice to remove any unbound antibody and resuspended in 700 µl buffer for analysis on a FACsCalibur flow cytometer (Becton Dickinson, Sunnyvale, CA, USA). Living cells were gated on a dot plot display of forward scatter versus side scatter, and CD14 was detected on the fluorescence-2 channel. An unstained aliquot of each cell subset was run in parallel with each positive sample in order to eliminate background fluorescence. Data analysis was performed using CellQuest software (Becton Dickinson).

2.2.4 DNA Techniques

DNA Isolation

DNA was isolated from 200 µl of whole blood by incubation in TNE lysis buffer (0.11 M sodium chloride, 55 mM Tris pH 8.0, 1.1 M EDTA pH 8.0, 0.55% sodium dodecyl sulphate) and 20 mg/ml Proteinase K (Roche, Mannheim, Germany) for 60 minutes at 37°C in a total volume of 600 µl. Buffer saturated phenol (Invitrogen) (450 µl) was added and the samples and vortexed for 10 minutes. After centrifugation at 12 000 x g for 5 minutes, the aqueous (top) layer was transferred to a fresh tube containing 450 µl of chloroform/isoamyl alcohol (50:1) and vortexed for 5 minutes. The centrifugation step was repeated, the aqueous layer transferred to a fresh eppendorf containing 40 µl of 3 M sodium acetate (pH 5.2), and 800 µl of 100% ethanol (-20°C) was added. After thorough mixing, the DNA was precipitated overnight at -20°C or at -70°C for 1 hour. The DNA was then collected by centrifugation at 12 000 x g at 0°C for 10 minutes. The pellet, washed

with 200 µl 80% ethanol, was allowed to air dry, and resuspended in 50 µl of nuclease-free H_2O.

Polymerase Chain Reaction (PCR)

Amplification of DNA samples was carried out by the polymerase chain reaction in a PTC-200 DNA Engine (MJ Research, MA, USA). PCR amplification was generally performed in a 50 µl total volume. All amplifications were primed by pairs of chemically synthesised 17 to 25-mer oligonucleotides designed using published DNA sequence (Entrez Nucleotide). The standard reaction mix is outlined in Table 2.1. Cycling parameters vary and thus are listed for individual PCR reactions.

Table 2.1 PCR reaction mixture.

Constituent	Final
10X *Taq* polymerase buffer (Promega)[a]	5 µl
Forward primer (100 µM)	0.2µM
Reverse primer (100 µM)	0.2µM
Each dNTP (Roche) (3 mM)	30µM
DMSO (Sigma)	4% (2µl)
Taq DNA polymerase	0.4 units
Genomic DNA	2 µl
Distilled water	Up to 50µl

[a]50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, and 1.5 mM $MgCl_2$.

Agarose Gel Electrophoresis

DNA products were separated by electrophoresis in 1-4% (w/v) agarose gels in TBE running buffer in horizontal gel electrophoresis tanks (Invitrogen). Gels were prepared by melting agarose (Invitrogen) in Tris-borate EDTA buffer (TBE) (0.04 M Tris borate, 0.001 M EDTA). The gel was cooled to 60°C, and ethidium bromide was added to a final concentration of 1.6 µg/ml. This was poured into the appropriate sized electrophoresis tank, and a well-forming comb was put in place. Once the gel was set, TBE buffer was added up to the level of the gel only, samples and appropriate molecular weight markers (Promega, Madison, WI, USA) were loaded and the gel allowed to run for 2 minutes at approximately 110 volts (depending on the distance between electrodes). Buffer was then added to cover the gel and the gel was electrophoresed for up to 90 minutes, until sufficient separation of DNA products was seen. DNA bands were visualised using a TFX-20.M ultraviolet transilluminator (Vilber Lourmat, France) and photographed using a Polaroid

GelCam (Polaroid, Cambridge, MA, USA) with Polaroid 665 (ASA 80) film, or a Kodak digital camera with Kodak digital science 1D 2.0.3 software.

2.2.5 Development of PCR-RFLP assays

Primer design

From the relevant Genbank database sequences, PCR primers were designed to flank the conserved regions of the polymorphisms under investigation (see Appendix A for relevant DNA sequences containing primer sites and restriction enzyme cut sites), giving an ideal size of PCR product between 150 to 600 base pairs. The primers were constructed to be between 15 and 30 base pairs in length, with GC content between 40 to 60%. Internal complementarity and 3' complementarity was avoided in order to avoid formation of internal secondary structure and production of primer dimers. Melting temperature of primers was approximated, and primers with melting temperatures between 50 to 68°C were used. The annealing temperature of pairs of primers was made to approximately match. Finally, Blast searches (http://www.ncbi.nlm.nih.gov/BLAST/) were performed in order to ensure that the designed primers were specific and would not anneal to genomic DNA outside the region to be amplified.

PCR conditions

The PCR amplification process was optimised with control DNA samples by using a range of annealing temperatures, generally from 54 to 62°C, in steps of 2°C. Further optimisation was achieved by performing dNTP curves (30 μM, 15 μM, 6 μM each dNTP), DMSO curves (0, 2, 4, 6, 8%), and primer concentration curves (1 μM, 0.5 μM, 0.2 μM each primer).

Restriction enzyme digestion and agarose gel electrophoresis

The Webcutter program (http://rna.lundberg.gu.se/cutter2/index.html) was used to identify the restriction enzyme most suitable for each assay. The ideal enzyme would cut the DNA when one allele is present, but not when the second allele is present. This enzyme would also have a constant restriction site within approximately 20 to 250 bp of the polymorphic site. This constant site would provide a control DNA fragment to confirm restriction enzyme activity. Restriction enzyme digestion was optimised using varying amounts of enzyme and varying digestion times (3 hr, 6 hr, 12 hr, overnight). The concentration of agarose gel used to visualise the restriction digestion product was selected on the basis of

the size of DNA fragments to be visualised and the size difference between these DNA fragments. Restriction digest or PCR products from assays with a small size difference between DNA fragments (~20 bp), such as the TNF-α -308 assay, were run in a low percentage gel (1.5 or 2%) in order to ensure maximum separation of these fragments. Assays where the PCR amplification yield is low and good resolution of DNA is needed required higher agarose gel concentrations (3 or 4%). See Appendix A for examples of PCR and restriction digest products separated by agarose gel electrophoresis and visualised by ethidium bromide staining.

2.2.6 RNA Techniques

Creating a Ribonuclease Free Environment

Ribonuclease free conditions were created in order to prevent RNase contamination and thus RNA degradation. Microbiological sterile technique was used, gloves were changed regularly, and the laboratory area used was treated with RNase away (Molecular BioProducts, San Diego, CA, USA). Reagents and utensils were set aside specifically for RNA work. Sterile disposable RNase free tips (Molecular BioProducts) and eppendorfs (Genex Laboratory Products, Northampton, UK) were used, and all solutions were made up with DEPC H_2O. All reaction steps and reagents were carried out or stored on ice where possible.

RNA Isolation

Total RNA was isolated from cells using a TRI reagent protocol. Cells were pelleted (300 x g, 10 minutes), resuspended in 1 ml TRI reagent (Sigma) per 5-10 $x10^6$ cells, and lysed by repetitive pipetting. The lysate was placed in a QIAshredder column (Qiagen, Sussex, UK) in 2 aliquots of 500µl and centrifuged at 12 000 x g for 2 min. The lysate was transferred to a 1.5 ml eppendorf and incubated at room temperature for 5 minutes. Chloroform, 200 µl per 1 ml TRIzol initially used, was then added, and the mixture was shaken vigorously for 15 seconds before incubation at room temperature for 2-3 minutes followed by centrifugation at 12 000 x g for 15 minutes at 2-8°C. This chloroform step was repeated to ensure all phenol was removed. The upper aqueous phase, containing the RNA, was then transferred to a fresh tube, 500 µl isopropanol per 1ml TRIzol used in the initial step was added, and the samples left at room temperature for 10 minutes followed by centrifugation at 12 000 x g for 10 minutes at 2-8°C. The RNA, now in a pellet on the sides and bottom of the tube, was washed with 1 ml 75% ethanol, air dried for 5-10 minutes, and

resuspended in 100µl nuclease-free water, prior to cleanup and DNase treatment.

RNA Cleanup and DNase treatment

RNA was cleaned up and DNase treated using the RNeasy Mini Kit and RNase-free DNase Set (Qiagen) according to the manufacturer's instructions. In brief, RNA in 100 µl nuclease free water was mixed with Buffer RLT (containing β-mercaptoethanol) and 96-100% ethanol before being passed through an RNeasy mini column by centrifugation at 10 000 x g for 15 seconds and the eluent discarded. The column was then washed with buffer RW1 and centrifuged, after which DNase solution (10 µl stock mixed with buffer RDD) was pipetted directly onto the column. The column was left at room temperature for 15 minutes, washed again with buffer RW1, and washed twice with buffer RPE, the second wash requiring a longer centrifugation time of 2 minutes. To elute the RNA, the column was transferred to a 1.5 ml collection tube, and 30 µl RNase free water was pipetted onto the column which was centrifuged at 10 000 x g for 1 minute. This step was then repeated, collecting the eluent in the same tube to give a final volume of 60 µl of total RNA. The RNA was then aliquoted and stored at –70°C. RNA concentration was checked by spectrophotometry, and if a higher concentration was needed, RNA was be precipitated and resuspended at the desired concentration.

RNA Precipitation

In order to precipitate RNA from solution, a 1/10 volume (of the starting RNA solution) of 3 M sodium acetate, pH 5.2 (0.3 M final concentration) was added, followed by 2.5 to 3 volumes of 96-100 % ethanol. The samples were mixed and frozen at –20°C for 30-45 minutes, then centrifuged at 18 000 x g for 20 minutes. The RNA pellet was washed with 75% ethanol, air dried, and resuspended in the required volume of nuclease free water.

Determination of RNA Concentration and Purity

RNA concentration was determined using a spectrophotometer. RNA samples were diluted 1:1000 with 10 mM Tris HCl, pH 7.5, and optical density (OD) readings taken at 230 nm, 260 nm, 270 nm, and 280 nm in a quartz cuvette using a UVmini-1240 spectrophotometer (Shimadzu, Kyoto, Japan). An OD reading of 1 measured at 260 nm corresponds to 40 µg/ml of single stranded RNA, relative to the dilution factor. The ratio of readings at 260 and 280 nm provides an estimate of protein contamination, with pure RNA preparations having an OD_{260}/OD_{280} value of 2.14. Protein contamination lowers this ratio by increasing the OD_{280} reading. Phenol contamination causes a peak at 270 nm, and the OD_{270} value

should be no greater than 0.8 of OD_{260}. Contaminating organics increase the OD_{230}/OD_{260} ratio, and the OD_{230} reading should be no greater than 0.5 of OD_{260} if the RNA preparation is free of such contaminants.

RNA Formaldehyde Agarose Gel Electrophoresis

RNA quality was determined by running a small RNA aliquot (~1µg) in a denaturing formaldehyde gel. A 1.8% gel was prepared by melting Seakem GTG Agarose (BMA, Rockland, ME, USA) in DEPC H_2O, 36 ml placed in a water bath at 60°C for 15 minutes, after which 9 ml formaldehyde (37%) and 5 ml 10X MOPS buffer (both Sigma) were added to give a final concentration of 1.3% agarose. The mixture was returned to the water bath for a further 5 minutes, mixed, poured into a horizontal gel rig that had been cleaned with hydrogen peroxide (1:10 dilution of 30% H_2O_2, Merck, NJ, USA), and allowed to solidify for 30 minutes.

Whilst the denaturing gel was solidifying, RNA, approximately 1 µg per sample to be visualised, was allowed to defrost slowly on ice. Loading dye [6.5 ml formamide (99.5%), 1.25 ml formaldehyde (37%), 2 ml MOPS buffer (10X), 0.4 ml 50% sucrose, 20 mg bromophenol blue, 20 mg xylene cyanol] was added to each RNA sample and an appropriate RNA marker (0.28-6.58kb) (Promega, Madison, WI, USA) in a 1:1 ratio with the volume of RNA. Samples were incubated at 65°C for 15 minutes, chilled on ice, and centrifuged for 5 seconds to deposit all fluid in the bottom of the microcentrifuge tube. The formaldehyde gel was run submerged in 1X MOPS buffer at 5 volts/cm between electrodes for 5 minutes before loading the RNA samples. Once the samples were loaded, the gel was run at 3-4 volts/cm between electrodes for approximately 2 hours, until the bromophenol blue had run 8 cm on the gel. The gel and direction of current was reversed at the halfway point (bromophenol blue 4 cm) as an alternative to constant buffer recirculation.

The gel was stained by constant circulation in 5 µg/ml ethidium bromide for 5 minutes, followed by destaining in DEPC H2O for 3 hours or overnight. RNA bands were visualised using a TFX-20.M ultraviolet transilluminator (Vilber Lourmat, France) and photographed using a Polaroid GelCam (Polaroid, Cambridge, MA, USA) with Polaroid 665 (ASA 80) film, or a Kodak digital camera with Kodak digital science 1D 2.0.3 software. Sharp ribosomal RNA (rRNA) bands (28S 4.7 kb and 18S 1.95 kb), with the 28S band approximately 2 fold the intensity of the 18S band, indicates good quality RNA, whilst degraded RNA shows up as diffuse and smeared rRNA bands or as a very low

molecular weight smear (RNA completely degraded). Genomic DNA contamination is indicated by high molecular weight bands running above the rRNA bands on the gel.

DNA-specific PCR of RNA samples

In order to confirm the lack of DNA contamination in RNA samples, in addition to visualisation by denaturing gel, PCR was performed on RNA samples using primers designed to cross an intron-exon border, thus only capable of amplifying genomic DNA. DNA contamination in RNA samples was therefore indicated if a product was seen following this PCR. Primers used annealed to the MTHFR gene (5'-TGAAGGAGAAGGTGTCTGCGGGA-3' and 5'-AGGACGGTGCGGTGAGAGTG-3'). The reaction mix is shown in Table 2.1 (Section 2.2.4), with the difference that 1 μM each primer was used. PCR conditions were 94°C 1 minute, 62°C 1 minute, and 72°C 1 minute for 40 cycles. PCR products were run in a 3% agarose gel as described in Section 2.2.4. Both a postive and negative control sample was amplified and run along with RNA samples to be tested.

Reverse Transcription of RNA

Reverse transcription (RT) PCR was performed in a total volume of 30μl. The RT PCR reaction mix consisted of 1X AMV RT Buffer (Promega) (50 mM Tris-HCl, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM spermidine), 0.2 mM each of deoxyribonucleoside triphosphates (Roche), 2.7 μM random hexamer primers (Promega), 5 units of avian myeloblastosis virus (AMV) reverse transcriptase enzyme (Promega), 10 mM DTT, 15% DMSO, 24 units RNaseOUT ribonuclease inhibitor (Invitrogen), and 4μl total RNA. The reaction was incubated at 37°C for 1 hour and the resulting cDNA was stored at –70°C.

2.2.7 ELISA Techniques

Enzyme-linked immunosorbent assays (ELISAs) were carried out using R&D Systems DuoSet ELISA Development Systems for human IL-6, TNF-α, IL-10, IL-1β, IL-4, IL-8, and IFN-γ. All antibodies and standards were diluted to the concentrations recommended in the individual protocols, and all buffers used were as recommmended by the manufacturers. The general protocol was followed for each assay according to the manufacturer's instructions.

CHAPTER 3

Investigation of the Effects of Protein C and Activated Protein C on Endotoxin Exposed Human Monocytes

3.1 INTRODUCTION

3.1.1 Lipopolysaccharide-Induced Sepsis

The inflammatory response to infection is initiated by the presence of infectious organisms, or by soluble products of these organisms such as Gram negative bacterial lipopolysaccharide (LPS) endotoxin, protein exotoxins (secreted toxins) from Gram positive bacteria, and cell wall glycopeptides such as teichoic acids and muramyl peptides. An exaggerated response to the presence of these factors can lead to systemic inflammatory response syndrome (SIRS), sepsis, or septic shock with multiorgan failure. Despite much research over the past three decades, no effective global therapy for these diseases has been identified. Septic shock, characterised by hypotension, inadequate organ perfusion, and multiple organ failure, carries a mortality rate of 10-15% in children and up to 40% in adults (Carcillo and Cunnion, 1997) and is the leading cause of death in hospital intensive care units. LPS, the most potent of the products mentioned above, is the cause of the greatest number of septic shock cases. Thus studies of monocyte recognition of LPS and its subsequent effect on these cells provide useful information about infection-induced inflammatory responses. LPS elicits a broad range of biological activities, interacting with many cellular and humoral elements, and mediating the host response to infection (Glauser *et al*, 1991) (Figure 3.1). However, sepsis or SIRS develop when these natural mechanisms for the maintenance of homeostasis break down (Rietschel *et al*, 1996). Despite the judicial use of antibiotics plus other adjuvants or therapies, effective treatment for severe endotoxaemia remains elusive, partly due to the difficulty of decreasing levels of free endotoxin in the circulation.

LPS is the principal component of the outer membrane of Gram negative bacteria and essential for bacterial viability. LPS molecules consist of an O-specific chain, a core oligosaccharide, and a lipid component (termed lipid A) (Rietschel *et al*, 1994). The O-specific chain, which varies considerably in structure across bacterial species, confers resistance to phagocytosis and bactericidal agents such as complement (Rietschel *et al*, 1996). The lipid A region, which is highly conserved across bacterial families, is thought

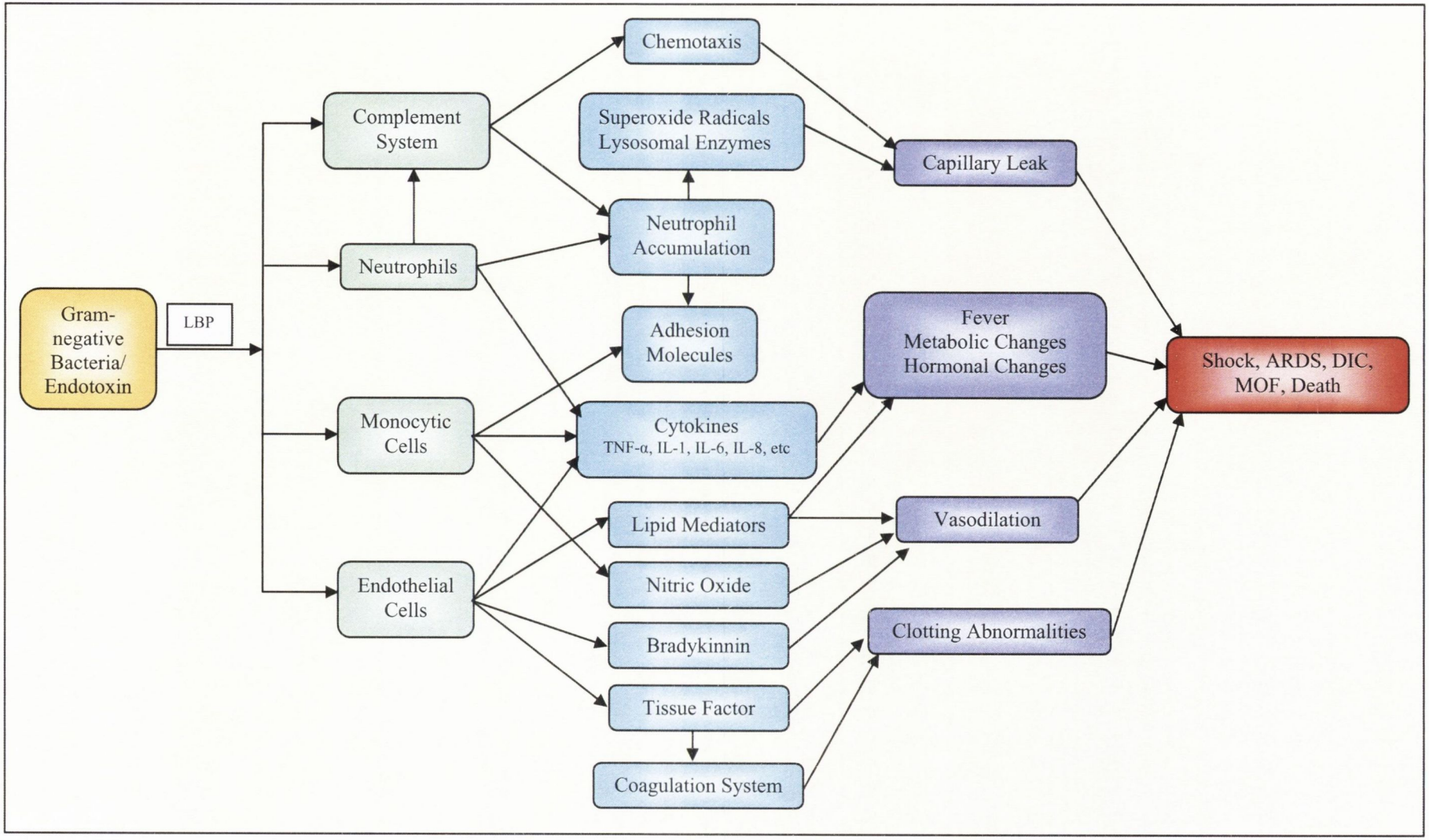

Figure 3.1 The systemic inflammatory cascade. LBP = LPS binding protein; ARDS = adult respiratory distress syndrome; DIC = disseminated intravascular coagulation; MOF = multiple organ failure.

to determine the endotoxic activities of LPS. It can reproduce the toxic effects of LPS in isolation. Synthetic lipid A, with no carbohydrate constituents, is as toxic as the naturally occurring molecule (Ulevitch and Tobias, 1995; Rietschel *et al*, 1994). Humans can be exposed to endotoxin by two methods. The first, and more widely understood method, is a result of a systemic or localised bacterial infection from an exogenous source, where bacteria or bacterial cell wall fragments cause an inflammatory response either locally or systemically. The second route of exposure occurs following bacterial translocation from the gut into the bloodstream. The gastrointestinal tract contains an indigenous non-pathogenic bacterial population which includes a number of Gram negative species. Thus fragments of the outer membrane, including endotoxin, are constantly being produced in the gut at low doses without harm to the host and are cleared by cells of the liver before reaching the circulation (Jacob *et al*, 1977; Van Leeuwen *et al*, 1994). However, if one or more of the host controls in this process fails, large quantities of bacteria or endotoxin can exit the gut *via* vascular or lymphatic channels and enter the circulation causing endotoxaemia (Van Leeuwen *et al*, 1994; van Deventer *et al*, 1988).

In the bloodstream, LPS and LPS-containing particles form complexes with LPS-binding protein (LBP), a 60 kDa plasma glycoprotein whose levels rise dramatically following an acute phase response (Schumann *et al*, 1990). Studies of LBP-deficient mice show that LBP is not required *in vivo* for the clearance of LPS from the circulation but is needed for the rapid induction of an inflammatory response to small amounts of LPS or Gram negative bacteria (Jack *et al*, 1997). The formation of the LBP-LPS complex facilitates delivery of LPS to CD14 (Wright *et al*, 1990), a cell surface receptor found on monocytes and macrophages. Studies of CD14-deficient mice and mice with CD14 over-expression demonstrate the requirement of CD14 for monocyte/macrophage activation by LPS (Haziot *et al*, 1995; Ferrero *et al*, 1993), however the absence of a transmembrane segment in CD14 suggests that CD14 is unlikely to be the LPS signalling molecule. This was confirmed by the discovery of Toll-like receptor 4 (TLR4), the missing link in the LPS-signalling pathway (Medzhitov *et al*, 1997; Poltorak *et al*, 1998; Qureshi *et al*, 1999; Hoshino *et al*, 1999). Binding of the LBP-LPS complex to cell surface CD14 and subsequent signalling *via* TLR4 causes a cascade of intracellular signalling events (Ulevitch and Tobias, 1994), leading to increased transcription factor expression and the production of various pro- and anti-inflammatory mediators (Mackman *et al*, 1991; Dentener *et al*, 1993). The main LPS signalling pathways and associated transcription factors are illustrated in Figure 3.2.

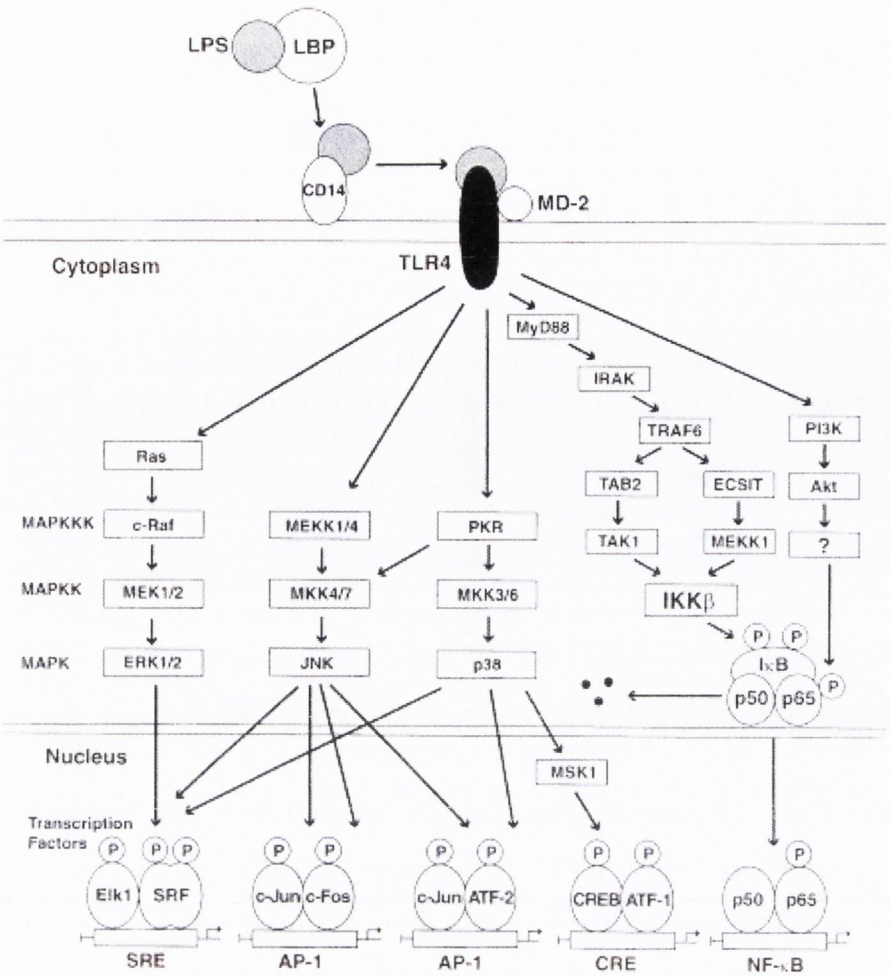

Figure 3.2 LPS signalling pathways in monocytes. The LPS/LBP complex binds to CD14, and LPS then interacts with TLR4/MD-2, stimulating various MAPK pathways including ERK, JNK, and p38. The IKK pathway is also activated via MyD88, IRAK, and TRAF6. Transcription factors such as Elk-1, c-Jun, c-Fos, ATF, SRF, CREB, and NF-κB are activated, and these bind to adjacent sites in LPS-inducible genes stimulating a wide range of gene expression. From (Guha and Mackman, 2001).

The systemic effects of LPS are principally mediated by macrophages as demonstrated by the transfer of endotoxin sensitive C3N/HeN macrophages into C3N/HeJ mice which are tolerant to otherwise lethal doses of endotoxin due to mutations in the TLR4 locus. This transfer causes these mice to become highly sensitive to the lethal effects of LPS (Freudenberg *et al*, 1986). The primary mediators of sepsis are TNF-α and IL-1β, which are produced rapidly and in large quantities by monocytes, macrophages, and other leukocytes in response to bacterial toxins. The major effects produced by bacterial toxins can be reproduced by the injection of TNF-α into animals, demonstrating the importance of this cytokine in sepsis (Tracey *et al*, 1986). These primary mediators lead to production of a vast range of factors including cytokines such as IL-6, G-CSF, GM-CSF, M-CSF, IL-10; chemokines including IL-8 and MCP-1; and receptors such as tissue factor and IL-2Rα. Production of numerous other humoral mediators is also stimulated by LPS, and activation

of the complement system and the coagulation system, along with production of nitric oxide (NO), ultimately leads to vasodilation, increased vascular permeability, circulatory collapse, the release of lysosomal enzymes and arachidonic acid metabolites, and the development of DIC, hypotension, and shock. However, the overwhelming systemic production of inflammatory cytokines is thought to be the principal element contributing to the life threatening organ failure and shock that distinguishes endotoxin-related sepsis.

3.1.2 Inflammation, Coagulation, and the Protein C Pathway

The host inflammatory and pro-coagulation responses to infection are unquestionably related. Inflammatory cytokines such as TNF-α, IL-1β, and IL-6 are capable of activating coagulation and inhibiting fibrinolysis (Conkling *et al*, 1988; Yan and Grinnell, 1993; Stouthard *et al*, 1996) as, for example, in the down-regulation of thrombomodulin and the endothelial protein C receptor (EPCR) expression by TNF-α (Conway and Rosenberg, 1988; Fukudome and Esmon, 1994) and the increase in macrophage pro-coagulant activity following IL-1β or TNF-α treatment (Schwager and Jungi, 1994). Conversely, the procoagulant protein thrombin is capable of stimulating inflammation, for example by enhancing LPS-induced inflammation (Hoffman and Cooper, 1995) or up-regulating ICAM-1 expression in monocytes (Clark *et al*, 2003), whilst TF-FVIIa complexes have been shown to induce a variety of pro-inflammatory responses in macrophages (Cunningham *et al*, 1999). Thus natural anticoagulants may play a role in modulating the host response to bacterial endotoxin. PC/APC, antithrombin, and TFPI decrease mortality rates in animal models of septic shock obtained by intravenous administration of endotoxin or of *Escherichia coli* (Emerson *et al*, 1989; Taylor *et al*, 1987; Creasey *et al*, 1993). In these studies, the elevation of natural anticoagulant levels decreased the inflammatory response in addition to the expected decrease in disseminated intravascular coagulation.

There is much evidence supporting involvement of the protein C pathway in the regulation of the inflammatory response to Gram-negative sepsis. Initially, it was shown that APC administration prevents the coagulopathic, hepatotoxic, and lethal effects of *Escherichia coli* infusion in the baboon, whilst the inhibition of protein C activation by the use of an antibody to protein C caused a more severe response to *E. coli* infusions (Taylor *et al*, 1987). The same group then demonstrated that the use of DEGR-Xa, an active site blocked factor Xa which inhibits thrombin generation, did not have an effect on the lethal effects of *E. coli* infusion, despite reducing the level of coagulation abnormalities seen in the animals

by the prevention of fibrin formation (Taylor *et al*, 1991). They therefore concluded that APC either blocks an early step in coagulation that contributes to organ damage or that APC blocks one or more components of the inflammatory response and thus prevents the associated shock and organ failure. Furthermore, APC was subsequently shown to reduce LPS-induced pulmonary vascular injury and LPS-induced pulmonary accumulation of leukocytes in rats (Murakami *et al*, 1996). Heparin plus ATIII, DEGR-Xa, or DIP-APC, an active-site blocked APC, did not prevent the vascular injury or leukocyte accumulation, although the first two inhibited LPS-induced coagulopathy suggesting that these anti-inflammatory properties of APC are independent of its inhibition of the pro-inflammatory clotting factor thrombin (Murakami *et al*, 1996). In addition, APC, but not DEGR-Xa or DIP-APC, inhibited the LPS-induced increase in plasma TNF-α levels measured in these rats (Murakami *et al*, 1997). Other studies found that APC treatment inhibits LPS-induced pro-inflammatory cytokine production in human monocytes (Grey *et al*, 1994; White *et al*, 2000b; Yuksel *et al*, 2002). Additionally, LPS-induced nuclear translocation of NF-κB, a critical transcription factor in LPS-induced pro-inflammatory cytokine production (Yao *et al*, 1997), is inhibited by APC (White *et al*, 2000b), whilst inhibition of both NF-κB and AP-1, also involved in the LPS-induced monocyte production of TNF-α, by APC has also been reported (Yuksel *et al*, 2002). Furthermore, blocking of the EPCR in baboons exposed to sub-lethal levels of *E. coli* causes a lethal response (Taylor *et al*, 2000). Animals receiving the EPCR-blocking antibody experienced consumptive coagulopathy, influx of neutrophils into the adrenal, renal, and hepatic microvasculature, and increased circulating levels of thrombin-antithrombin complex, IL-6, and IL-8. These results lead to the conclusion that binding of PC/APC to the EPCR contributes significantly to the negative regulation of the coagulopathic and inflammatory response to *E. coli* infusion. Finally, both APC and its zymogen PC function to inhibit neutrophil and monocyte chemotaxis triggered by a variety of stimuli including IL-8 (Sturn *et al*, 2003), indicating a potential therapeutic role for PC and APC in monocyte- and neutrophil-mediated diseases such as acute respiratory distress syndrome (ARDS), SIRS, or sepsis.

The most compelling evidence for the critical involvement of the protein C pathway in the modulation of the monocyte response to LPS comes from clinical trials of PC or APC treatment in meningococcaemia or other forms of sepsis. A series of clinical studies suggested a trend towards improved survival of septic patients treated with PC zymogen (Smith *et al*, 1997; Hartman *et al*, 1998; Smith and White, 1999; White *et al*, 2000a). These positive results were confirmed in a randomised, double-blind, placebo-controlled,

multicentre phase 3 trial (PROWESS), where a total of 850 patients with systemic inflammation and organ failure were treated with recombinant human APC (rhAPC), with 840 patients in the placebo group. In this study, treatment with rhAPC was associated with a reduction in the relative risk of death of 19.4% (95% CI 6.6-30.5), and an absolute reduction in the risk of death of 6.1% (p=0.005) (Bernard *et al*, 2001) despite an increase in serious bleeding in the rhAPC group.

Coagulation factors, especially thrombin, and other proteases regulate cell responses to tissue injury or vascular leakage by cleavage of platelet and endothelial cell surface protease-activated receptors (PARs) leading to cellular activation and potentiation of the inflammatory response (Coughlan, 2001). Although originally described in platelets and endothelial cells, PARs have been detected in many other cell types, particularly those involved in inflammatory processes (Vergnolle, 2003). PARs are a family of homologous G-protein-coupled receptors that are activated by proteolytic cleavage rather than ligand binding (Vu *et al*, 1991). Of the four PARs identified to date, three are cleaved by thrombin while PAR-2 can be activated by trypsin and mast cell trypase (Coughlan, 2001). PAR-4 can also be activated by trypsin. Riewald *et al* recently demonstrated the use of EPCR by APC as a co-receptor for cleavage of PAR-1 on endothelial cells (Riewald *et al*, 2002). They found, through gene profiling of the effect of APC treatment on endothelial cells, that PAR-1 signalling could account for all APC induced genes in the endothelial cell, including the monocyte chemoattractant protein 1 (MCP-1) gene which was selectively induced by PAR-1 but not PAR-2 activation. To exclude thrombin as a potential contaminant in the APC preparation, they performed control experiments with hirudin as a direct inactivator of thrombin. Similarly, Mosnier and Griffin reported that the inhibition of apoptosis of endothelial cells by APC requires PAR-1 and EPCR (Mosnier and Griffin, 2003) and Cheng *et al* reported that APC protected human brain endothelium from apoptosis, dependent on the presence of EPCR and PAR-1 (Cheng *et al*, 2003). Thus the anti-apoptotic activity of APC in endothelial cells appears to be mediated by cleavage of PAR-1 by the APC-EPCR complex. However, activation of PAR-1 is unlikely to account for the other cellular responses associated with APC or PC treatment. PAR-1 and PAR-2 agonists have no effect on PC or APC induced inhibition of eosinophil or neutrophil chemotaxis (Feistritzer *et al*, 2003; Sturn *et al*, 2003) and whilst APC inhibits pro-inflammatory cytokine release and NF-κB translocation in monocytes (Section 3.1.2), activation of PAR-1 on monocytes and macrophages leads to the release of inflammatory cytokines such as IL-1β and IL-6 (Naldini *et al*, 1998; Naldini *et al*, 2000) and both

thrombin and PAR-1 activation peptide stimulate IL-8 release in monocytic U937 cells (Suk and Cha, 1999).

The molecular mechanisms responsible for the apparent anti-inflammatory properties of APC are not fully understood. It appears that PC/APC binds to monocytes and endothelial cells and thus prevents the overproduction of cytokines such as IL-1, TNF-α, IL-8, and IL-6 which cause many of the sequelae associated with Gram-negative septic shock. However, it is thought that this suppression of expression is not a generalised effect where all aspects of cell activity are blocked. Looking at the effect of rhAPC on endothelial cell gene expression, Joyce *et al* showed that most genes modulated by rhAPC treatment were either involved in the inflammatory response or cell survival (Joyce *et al*, 2001), indicating specific pathways in the endothelial cell system that are affected by rhAPC treatment.

3.1.3 Microarray Technology for Gene Expression Analysis

Although mRNA is not the final product of a gene, and modulation of eukaryotic gene regulation also involves mRNA stability, post-translational modifications, regulation of protein transport, and protein degradation, transcription is the first step in gene expression and plays an important role in gene regulation. Thus, information about gene transcript levels is important in understanding the processes occurring in a cell. Additionally, the analysis of transcript levels is currently more technologically advanced, cheaper, and can be done in a more high-throughput way than the direct measurement of protein levels. Many methods have been used to detect and quantitate differences in gene expression levels, from reverse Northern blots on filters detected using radioactive probes, to a wide range of methods such as differential hybridisation screening, subtractive cDNA library construction, ribonuclease protection, sequencing of cDNA libraries (the expressed sequence tag, EST, approach), reverse transcriptase-polymerase chain reaction (RT-PCR), differential display, serial analysis of gene expression (SAGE), representational differential analysis (RDA), and suppression subtractive hybridisation (SSH). However, microarray technology is the method of choice for quantitative analysis of the expression of thousands of genes as it is a high throughput method and allows true parallelism, miniaturisation, multiplexing, and automation (Schena *et al*, 1998).

Microarray technology is based on the idea, proposed almost twenty five years ago by Ed Southern, that 'labelled nucleic acids could be used to interrogate nucleic acid molecules

attached to a solid support' (Southern, 1975). A microarray is made by arraying cDNA targets or oligonucleotides onto a glass slide or membrane. These are then probed by fluorescently labelled cDNAs derived from RNA pools from test and reference cells. The relative abundance of message in the test cell sample is determined by direct comparison of fluorescent signal with that from the reference sample for each gene represented in the array. In this way, it is possible to identify genes whose expression levels differ between the two samples. This method provides high detection sensitivity and specificity as a result of the mutual selectivity between complementary strands of nucleic acids. Expression changes of twofold or greater have been shown to be reproducibly detectable in microarray experiments (Iyer, 1999). With microarray analysis, it is possible to study the RNA expression profile of tens of thousands of genes in a single experiment. A 1×3 inch glass slide can accommodate up to 25 000 individual spots, although arrays currently in use generally represent 5000 to 10000 genes.

The role of APC in modulating coagulation in the microcirculation through inhibition of thrombin generation has been studied in detail. However, the full extent of APC and PC action on monocyte function has not been fully elucidated. The use of microarray analysis to determine the effect of APC/PC on stimulated and unstimulated monocytes provides a powerful tool to allow the monitoring of gene expression of a significant portion of the genome including signal transduction pathways, the inflammatory pathway, the apoptosis pathway and other relevant biological systems, providing valuable information for the determination of the mechanism of APC action in the treatment of sepsis.

3.1.4 Aims

The process by which PC/APC exerts its anti-inflammatory effects is not fully understood, nor is the scope of the effect of this protein on the inflammatory response. However, much evidence points to a modulatory effect of APC or its zymogen PC on the activated monocyte. Binding of PC/APC to the monocyte appears to regulate intracellular signalling and the monocytic response to inflammatory stimuli. In order to fully understand the mechanism of action by PC/APC, an overall view of the effect of this protein on the gene expression profile of the activated and unactivated monocyte is critical. We therefore investigated the gene expression profile of a monocyte cell line after stimulation with LPS and looked at the effect of PC treatment on this profile by microarray analysis. We also investigated the effect of PC on the gene expression profile of unstimulated monocytes. To

validate the results obtained from the microarray gene expression profile investigation, we studied the expression of a range of cytokine genes under these treatment conditions by real-time PCR, and we determined the protein expression of these cytokines by ELISA. In addition, we investigated both gene expression, by real-time PCR, and protein expression, by ELISA, of these cytokine genes in primary human monocytes in response to LPS and PC treatment. PC must be activated for it to carry out its anticoagulant activities. In order to investigate the requirement for PC activation in the induction of its anti-inflammatory effects, a small scale investigation of the effect of APC treatment on both LPS-stimulated primary monocytes and THP-1 cells was undertaken. Finally, the viability and proliferation of the monocytes were investigated in order to ensure that all treatment conditions were non-toxic to the cells.

3.2 MATERIALS AND METHODS

3.2.1 THP-1 Cell LPS and PC Treatment for Gene Profile Analysis by Microarray Technology

The tissue culture routine for growing up the THP-1 cell line is described in Section 2.1. THP-1 cells were counted and resuspended in cell culture medium [RPMI 1640 supplemented with 10% foetal calf serum (Gibco), 2 mM L-glutamine (Gibco), 50 U/ml penicillin (Gibco), and 50 µg/ml streptomycin (Gibco)] at a concentration of 1 million cells per ml. Aliquots of 10 ml of cell suspension were plated in a six well plate, with a total of 6 wells being used. Three separate experiments and RNA isolations were carried out to provide RNA for microarray analysis: untreated (R) *versus* LPS (10 ng/ml) treated (LPS), untreated (R) *versus* PC (10 µg/ml) treated (PC), and PC pre-treated/LPS treated (LPS + PC) *versus* LPS treated (LPS). In each experiment, 3 wells of 10 million cells were used per sample (i.e. RNA from 30 million cells was extracted per sample). Pre-treatment with PC was for 30 minutes at 37°C, followed by addition of LPS with incubation for another 90 minutes before RNA extraction. Those samples not receiving LPS, PC, or both received an equal volume of PBS. RNA extraction, DNase I treatment, and determination of RNA quality and quantity were performed as outlined in Section 2.2.6. Any remaining RNA not used for microarray experiments was stored for TaqMan analysis in order to confirm findings resulting from the microarray experiments. Similarly, in each experiment, cell supernatant was stored for future ELISA analysis.

3.2.2 Microarray Experiments for Determination of the Gene Expression Profile of Untreated, LPS Treated, PC Treated, and LPS + PC Treated THP-1 Cells

Labelling, Hybridisation, and Scanning for Microarray Experiments

For each sample, 100 µg of RNA was sent to MWG Biotech AG (Ebersberg, Germany) for labelling, hybridisation, and scanning. MWG Biotech carries out all steps in a controlled environment using highly optimised conditions and experienced personnel, thus providing results of a higher quality than could be achieved if these steps were carried out in our routine laboratory. Scanning results were returned to us for analysis. Three microarray experiments were performed, where the 2 samples to be compared were differentially labelled by reverse transcription of RNA into cDNA incorporating either Cyanine-3-dCTP or Cyanine-5-dCTP and hybridised to the same microarray. As mentioned in Section 3.2.2

above, the three experiments were as follows: R *versus* LPS, R *versus* PC, L+PC *versus* LPS. Samples were hybridised to the MWG Human 10k Array A, a glass slide spotted with 50mer oligonucleotide probes representing 9850 genes, 30 replicated spots, and 104 Arabidopsis controls. The array also contained 384 empty wells, to be used for determination of background fluorescence. All steps were performed as described in the MWG Array Manual which can be downloaded at http://ecom2.mwgdna.com/services/array/download.tcl.

Microarray Analysis

MWG Biotech scanned the microarrays with a confocal scanning instrument containing two tuned lasers that excite Cyanine dyes at the appropriate wavelength (Affymetrix 428 Array Scanner, Affymetrix, CA, USA). The arrays were scanned at three different photomultiplier (PMT) gain settings, producing 16-bit tiff images that were used for analysis. ImaGene™ (BioDiscovery Inc, CA, USA) image analysis software was used to analyse the microarray images with pixel selection algorithms used to determine signal and background intensities for each individual spot. The image analysis data was then read into MAVI 2.6 software (MWG Biotech) for further processing. The raw background intensities were subtracted from the raw signal intensities to obtain raw intensity values corrected for background for each spot at each PMT gain setting.

Signal saturation occurs if a spot is bright and the PMT setting is high. From a certain brightness onwards a scanner will give out its highest possible value for the brightness of a spot. With 16-bit converters, this maximum value is 65536. Signal deterioration occurs if a spot is dark and the PMT setting is low causing the photomultiplier to work in a non-linear range thus causing the signal to be underestimated. In order to eliminate values that are saturated or deteriorated, MAVI made a line connecting the intensity values for one spot over the different amplifications measured and calculated the amplification value closest to 40% of the span of amplification values. The amplification value chosen is generally the middle of the 3 values recorded (35, 45, and 55). MAVI then fitted a straight line to all spots that probably didn't have saturation or deterioration (generally those with values between 40000 and 100) and calculated the average slope for both Cy-3 and Cy-5. The intensities of all spots were then gone through separately. To check for saturation, the slope between the intensities of the 2 highest amplification settings was compared with the average slope. If the difference was greater than 30%, the intensity of the 2 higher amplifications was removed. The slope of the next 2 highest amplifications was checked in

the same way, and this was done consecutively until one slope fulfilled the criterion of less than 30% difference to the average slope. The same was done consecutively from lower amplifications upwards to eliminate intensities with signal deterioration. To calculate intensities, the surviving intensities were taken and a linear regression of the log signals was performed. From the fitted line the intensity at the previously determined amplification was calculated, providing the <slide>.Ic3 and <slide>.Ic5 intensity value for each spot in the array.

Normalisation is performed to eliminate differences in the brightness of the Cy-3 and Cy-5 images (caused by effects such as sample preparation, labelling efficiency, scanning differences and others) which result in skewing of the ratio histogram (histogram of all ratios from the slide). The idea is to normalise the ratio of each spot so that the peak of the ratio histogram lies at 1. This is done by multiplying all ratios by a factor called the normalisation parameter (np). To get the np, the log average of both Cy-3 and Cy-5 intensities were calculated and a factor was calculated to multiply all Cy-3 intensities to make the Cy-3 log average the same as the Cy-5 log average. Intensities are also normalised, especially if comparing two different slides. In this case, the global intensities of the two slides are likely to vary, making it impossible to compare the intensities from the two. The intensities of each spot were normalised with respect to their log mean, chosen instead of mean as it is a more stable and reliable value, and does not depend so heavily on outliers with high intensities. Normalisation of intensities does not move the peak of the intensities histogram to 1, but the normalisation leads to a more reliable comparison of slides than taking raw values or values normalised by their average.

$$\text{normalised ratio} = \frac{\text{ratio before normalisation (r)}}{\exp[\text{average of ln r}]}$$

exp = e to the power of
average of ln r = is the average of the natural log of all the ratios

$$\text{normalised intensity} = \frac{\text{intensity before normalisation (I)}}{\exp[\text{average of ln I}]}$$

exp = e to the power of
average of ln I = is the average of the natural log of all the intensities for Cy-3 or Cy-5

.Ic3pc (normalised Cy-3 intensity) = .Ic3/exp [av ln I (Cy-3)]

.Ic5pc (normalised Cy-5 intensity) = .Ic5/exp [av ln I (Cy-5)]

.rat (normalised ratio) = .Ic3pc/.Ic5pc = (.Ic3/exp [av ln I (Cy-3)])/(.Ic5/[av ln I (Cy-5)])
.rat (normalised ratio) = .Ic3/.Ic5 x exp [av ln I (Cy-5)]/exp [av ln I (Cy-3)] = .Ic3/.Ic5 x 1/exp[av ln r] = .Ic3/.Ic5 x np

normalisation parameter (np) = 1/exp[av ln r] = exp [av ln I (Cy-5)]/exp [av ln I (Cy-3)]
.nIc3 = .Ic3 x np
.rat (normalised ratio) = .nIc3/.Ic5

These calculations provided (where <slide> is the microarray slide name):

<slide>.nIc3	(calculated Cy-3 signal intensity normalised with respect to Cy-5)
<slide>.rat	(normalised ratio)
<slide>.Ic3pc	(calculated Cy-3 intensity normalised with respect to its log mean)
<slide>.Ic5pc	(calculated Cy-5 intensity normalised with respect to its log mean)

Other information contained in the output file included signal to background values for each spot at amplification 45 that were calculated by dividing the raw signal by the raw background values. The MAVI results files were then imported into Excel sheets, thus combining the array data with gene description data. These sheets therefore contained all raw data, normalised and background corrected intensities, and ratio values. GeneSight (MWG Biotech) was used to visualise the data by creating ratio histograms and scatter plots of intensities.

Signals from empty wells included in the microarrays were used to eliminate genes with no expression in either sample hybridised to the array. The signal detected in these empty wells was due to slight autofluorescence from the buffers used in the spotting process or slight signal contamination by dust particles. The data from the empty wells was disregarded other than to check that all signals were low, and to set a cut off intensity value for no expression in the spots containing oligonucleotides representing genes. This cut off point was set twofold above the highest empty well signal (in all slides approximately 150 bytes/pixel), thus at approximately 300 bytes/pixel.

Normalisation of data from the two samples was performed using the data from all spots, a method which is particularly effective where the ratio data is in a roughly normal distribution and has been obtained from a large number of spots. Normalising on all spots relies on the assumption that each cell will have approximately the same amount of RNA

in total and that the two phenotypes have general similarities, which was the case with the large majority of genes in the arrays analysed. Therefore, although housekeeping genes were included in the arrays, they were not used to normalise the array data as it was not known exactly how they would perform in the exact phenotypes studied in the arrays and it was possible that their expression would vary depending on the treatment conditions of the cells. Another problem with the use of housekeeping genes for normalisation is that a large dataset (10000 genes) is being modified based upon the results of a small dataset (30 housekeeping genes), meaning that a small change in the housekeeping genes could have a major knock on effect on the data received from the 10000 other spots in the array.

3.2.3 Monocyte LPS and PC/APC Treatment for Cytokine Gene Expression Analysis by TaqMan and ELISA Technology

The isolation procedure for obtaining primary human monocytes is described in Section 2.2 and the tissue culture routine for growing up the THP-1 cell line is described in Section 2.1. Primary monocytes diluted in RPMI 1640 (Gibco, Invitrogen Ltd, Paisley, UK) with 10% autologous serum, or THP-1 cells diluted in cell culture medium [RPMI 1640 supplemented with 10% foetal calf serum (Gibco), 2mM L-glutamine (Gibco), 50U/ml penicillin (Gibco), and 50 µg/ml streptomycin (Gibco)], were aliquoted at 1 million monocytes per well in a 24 well microplate. Where primary monocytes were used, the cells were incubated overnight at 37°C to allow the cells to recover from any stimulation caused by the isolation procedure. Cells were then pre-incubated for 30 minutes with the appropriate concentration of APC or PC (generally from 200 µg/ml to 10 µg/ml), with an equal volume of PBS added to the wells not receiving APC or PC. After the pre-incubation, the appropriate amount of LPS (from 10 µg/ml to 10 ng/ml) (Sigma-Aldrich) was added to the appropriate wells, with an equal volume of PBS (Gibco) added to the wells that were not receiving LPS. After a 1.5, 4, 20, or 24 hour incubation period at 37°C in 5% CO_2 the contents of each well were removed and centrifuged at 400 x g for 5 minutes. The supernatant was removed and stored at –70°C for subsequent ELISA analysis (Section 2.3). RNA isolation was immediately performed on pelleted cells (Section 2.4).

To ensure no effect of thrombin on APC/PC treatment results, THP-1 cells ($1x10^6$/ml) were treated with hirudin (20 U/ml = 2 µg/ml = ~300 nM) and APC or PC (50 or 20 µg/ml). After 30 minutes LPS was added (10 ng/ml) and cells were incubated 4 hours at 37°C in 5% CO_2. Negative and LPS-only controls, both hirudin treated, were also included.

To verify the effect of APC/PC treatment on LPS stimulated monocytes without APC/PC preincubation or with LPS preincubation, a time course experiment was performed. THP-1 cells ($1x10^6$/ml) were untreated, LPS (10 ng/ml) treated, or LPS treated with APC or PC (50 µg/ml) added at varying time points (APC/PC addition time relative to LPS addition = -30 minutes, -15 minutes, 0 minutes, +15 minutes, +30 minutes, +60 minutes). Four hours after LPS addition, the contents of each well were removed, pelleted and the supernatant was removed for subsequent ELISA analysis (Section 2.3).

3.2.4 Real-Time PCR to Quantify Relative Cytokine Gene Expression in LPS and PC/APC Treated Monocytes

Preparation of cDNA

Each RNA sample, 4 µg, was reverse transcribed in a total reaction volume of 30 µl, as described in Section 2.2.6. As controls, 'no amplification' and 'no template' samples were also prepared by omitting AMV reverse transcriptase or template RNA respectively.

Real-time PCR Analysis of Relative Gene Expression Using TaqMan Technology

All mRNA quantitation analysis was carried out using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). To quantify the relative expression of a range of genes, Pre-Developed TaqMan Assay Reagents (PDARs) for IL-6, and IL-8, along with the TaqMan® Cytokine Gene Expression Plate I (all Applied Biosystems) were used for real-time PCR analysis. The PDARs consist of a mixture of pre-optimised concentrations of gene specific forward and reverse primers, along with 5'-FAM-labelled/3'-non-fluorescently-quenched (MGB) gene specific probes. The Cytokine Gene Expression Plate consists of a MicroAmp Optical 96 well reaction plate with 12 columns, each made up of 8 identical wells containing gene specific forward and reverse primers and 5'-FAM-labelled/3'-TAMRA-quenched gene specific probes for the assay of one cytokine (Figure 3.3). The 12 different columns contain primers and probes specific to 12 different cytokine genes, and all 96 wells also contain primers and VIC-labelled/TAMRA quenched probes specific to the 18S rRNA gene.

	1	2	3	4	5	6	7	8	9	10	11	12
A	IL-α Calibrator (1)	IL-1β Calibrator (2)	IL-2 Calibrator (3)	IL-4 Calibrator (4)	IL-5 Calibrator (5)	IL-8 Calibrator (6)	IL-10 Calibrator (7)	IL-12p35 Calibrator (8)	IL-12p40 Calibrator (9)	IL-15 Calibrator (10)	IFNγ Calibrator (11)	TNFα Calibrator (12)
B	IL-α Calibrator (13)	IL-1β Calibrator (14)	IL-2 Calibrator (15)	IL-4 Calibrator (16)	IL-5 Calibrator (17)	IL-8 Calibrator (18)	IL-10 Calibrator (19)	IL-12p35 Calibrator (20)	IL-12p40 Calibrator (21)	IL-15 Calibrator (22)	IFNγ Calibrator (23)	TNFα Calibrator (24)
C	IL-α Sample 1 (25)	IL-1β Sample 1 (26)	IL-2 Sample 1 (27)	IL-4 Sample 1 (28)	IL-5 Sample 1 (29)	IL-8 Sample 1 (30)	IL-10 Sample 1 (31)	IL-12p35 Sample 1 (32)	IL-12p40 Sample 1 (33)	IL-15 Sample 1 (34)	IFNγ Sample 1 (35)	TNFα Sample 1 (36)
D	IL-α Sample 1 (37)	IL-1β Sample 1 (38)	IL-2 Sample 1 (39)	IL-4 Sample 1 (40)	IL-5 Sample 1 (41)	IL-8 Sample 1 (42)	IL-10 Sample 1 (43)	IL-12p35 Sample 1 (44)	IL-12p40 Sample 1 (45)	IL-15 Sample 1 (46)	IFNγ Sample 1 (47)	TNFα Sample 1 (48)
E	IL-α Sample 2 (49)	IL-1β Sample 2 (50)	IL-2 Sample 2 (51)	IL-4 Sample 2 (52)	IL-5 Sample 2 (53)	IL-8 Sample 2 (54)	IL-10 Sample 2 (55)	IL-12p35 Sample 2 (56)	IL-12p40 Sample 2 (57)	IL-15 Sample 2 (58)	IFNγ Sample 2 (59)	TNFα Sample 2 (60)
F	IL-α Sample 2 (61)	IL-1β Sample 2 (62)	IL-2 Sample 2 (63)	IL-4 Sample 2 (64)	IL-5 Sample 2 (65)	IL-8 Sample 2 (66)	IL-10 Sample 2 (67)	IL-12p35 Sample 2 (68)	IL-12p40 Sample 2 (69)	IL-15 Sample 2 (70)	IFNγ Sample 2 (71)	TNFα Sample 2 (72)
G	IL-α Sample 3 (73)	IL-1β Sample 3 (74)	IL-2 Sample 3 (75)	IL-4 Sample 3 (76)	IL-5 Sample 3 (77)	IL-8 Sample 3 (78)	IL-10 Sample 3 (79)	IL-12p35 Sample 3 (80)	IL-12p40 Sample 3 (81)	IL-15 Sample 3 (82)	IFNγ Sample 3 (83)	TNFα Sample 3 (84)
H	IL-α Sample 3 (85)	IL-1β Sample 3 (86)	IL-2 Sample 3 (87)	IL-4 Sample 3 (88)	IL-5 Sample 3 (89)	IL-8 Sample 3 (90)	IL-10 Sample 3 (91)	IL-12p35 Sample 3 (92)	IL-12p40 Sample 3 (93)	IL-15 Sample 3 (94)	IFNγ Sample 3 (95)	TNFα Sample 3 (96)

Figure 3.3 TaqMan Cytokine Gene Expression Plate I Configuration.

To investigate the levels of gene expression using TaqMan technology, a reference must be used to normalise experimental results, thus correcting for varying amounts of target cDNA in different wells or samples. The reference can be an endogenous control (one that is present in each experimental sample isolated) or an exogenous control (one that is added to each sample isolated). The Cytokine Gene Plate contains pre-optimised concentrations of primers and probes for 18S rRNA as the endogenous control. We therefore chose to use this gene as the endogenous control for the PDAR assays too, using the 18S rRNA MGB PDAR (Applied Biosystems), which consists of pre-optimised concentrations of forward and reverse primers and a 5'-VIC-labelled MGB (non-fluorescently quenched) probe, all specific to the 18S rRNA gene. We also used a GAPDH endogenous control to verify results obtained in these PDAR assays (also Applied Biosystems). The choice of the most suitable method for analysis of relative expression, either by standard curve or by comparative C_T method, depends on the relative amplification efficiencies of both reactions. It is also necessary to determine whether the amplification of the control can be performed in the same reaction as the amplification of the target gene (multiplexing), depending on whether the two reactions interfere with one another. To select between multiplexing or single tubes, and between standard curve or comparative C_T methods,

template titration standard curves were prepared for both target and control genes in separate tubes and also both reactions in the same tube (multiplexed). A 10-fold serial dilution of LPS-treated human monocyte cDNA was prepared (from x 1 to x 0.0001). The reaction mix, set up in triplicate, consisted of 2 µl of the appropriate target cDNA concentration, 12.5 µl TaqMan Universal PCR Master Mix, 1.25 µl IL-6 MGB PDAR (Applied Biosystems) and/or 1.25 µl endogenous control MGB PDAR, made up to a 25 µl final volume with nuclease free H_2O. To control for contamination and amplification specificity, 'no template' (NTC) and 'no amplification' (NAC, no reverse transcriptase) controls were also prepared. Thermal cycling conditions used were 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. After the real-time PCR run, the baseline was corrected and threshold level was adjusted for each amplification curve in order to minimise any background fluorescence. A relative standard curve of log dilution values against threshold cycle (C_T) value [the cycle at which a statistically significant increase in the background-normalised fluorescence emission intensity (ΔR_n) is first detected] was constructed for target and control both from the single tube reactions and from the multiplex reaction. If the difference between the single tube target and control standard curve slope is less than 0.1, then the comparative C_T method can be used, however, if the difference in slopes is greater than 0.1, then analysis must be carried out by the standard curve method. If the slope of the target standard curve in the single tube is equal to the slope of the target in the multiplex reaction, and similarly for the control, then the multiplex method may be considered for use, if not then FAM- and VIC-specific reactions must take place in separate tubes.

The standard curve method and single tube reactions were chosen for real-time PCR analysis of IL-6, IL-8, or TNF-α gene expression in monocytes treated under conditions detailed in Section 3.2.3. Each sample was analysed in triplicate along with an appropriate standard curve consisting of serial dilutions of an LPS treated cDNA sample (each concentration also in triplicate). The reaction mix consisted of 2 µl of the appropriate target samples or standard sample cDNA, 12.5 µl TaqMan Universal PCR Master Mix, 1.25 µl target MGB PDAR or 1.25 µl endogenous control MGB PDAR, made up to a 25 µl final volume with nuclease free H_2O. Cycling conditions were as described above. Relative concentrations were calculated from the standard curve using the equation [(sample C_T value – b)/m] where m is the slope of the line, and b is the y-intercept. The relative amount of target was divided by the relative amount of endogenous control in order to obtain the

normalised amount of target expression. Generally, the cDNA sample with the lowest gene expression was designated as the calibrator for comparison of gene expression levels.

The Cytokine Gene Plate allows for the relative quantification in duplicate of the expression of 12 different cytokine genes in four cDNA samples (see Figure 3.3). Four reaction mixes were prepared (one per sample), each consisting of 700 µl TaqMan Universal PCR Master Mix, 588 µl nuclease-free H_2O, and 122 µl cDNA (~15 µg) from one of the 4 samples to be analysed. 50 µl of reaction mix was then transferred into each appropriate well in the Cytokine Plate: reaction mix for sample 1 into rows A and B, mix for sample 2 into rows C and D, sample 3 into rows E and F, and sample 4 into rows G and H. The final amount of cDNA per well was approximately 500 ng. Cycling conditions were as described above. After the real-time PCR run, the baseline was corrected and threshold level was adjusted for each amplification curve in order to minimise any background fluorescence. Relative quantification of cytokine gene expression was calculated by the comparative C_T method. In brief, ΔC_T is calculated by subtracting VIC C_T from FAM C_T. Average ΔC_T values for duplicate wells were obtained, and the average ΔC_T of the calibrator sample (generally the sample with the lowest gene expression) was subtracted from the average $\Delta\Delta C_T$ value of the other samples to obtain $\Delta\Delta C_T$ values. To obtain the relative quantification values, the $\Delta\Delta C_T$ value was then entered into the following equation: $2^{-\Delta\Delta C_T}$, to obtain values of gene expression relative to the expression of the calibrator sample.

3.2.5 ELISA to Quantify Relative Cytokine Gene Expression in LPS and PC or APC Treated Monocytes

ELISAs were carried out on tissue culture supernatants as described in Section 2.2.7 in order to quantify levels of secreted cytokines by primary monocytes or THP-1 cells untreated or treated with varying amounts and combinations of LPS, APC, and PC for varying periods of time (see Section 3.2.3).

3.3 RESULTS

3.3.1 Determination of Quality of RNA Samples Used for Microarray Analysis

RNA Quality Determination: Denaturing Formaldehyde Agarose Gel Electrophoresis and Bioanalyzer Total RNA Characterisation

In order to check the quality of the RNA isolated for array analysis, a small amount of each RNA sample along with an RNA marker ladder was electrophoresed in a denaturing formaldehyde agarose gel as described in Section 2.2.6. The ribosomal RNA (rRNA) bands of 4.7 kb (28S rRNA) and 1.95 kb (18S) were clearly visible in the gel (Figure 3.4A). The RNA was not degraded as indicated by sharp bands that were not smeared or diffuse and by the approximately 2 fold higher intensity of the 28S band compared to the 18S band. All RNA samples for microarray analysis were also analysed in an Agilent Technologies 2100 Bioanalyzer by MWG Biotech prior to cDNA labelling, also showing non-degraded RNA free of genomic DNA or other contaminants (Figure 3.4B and 3.5).

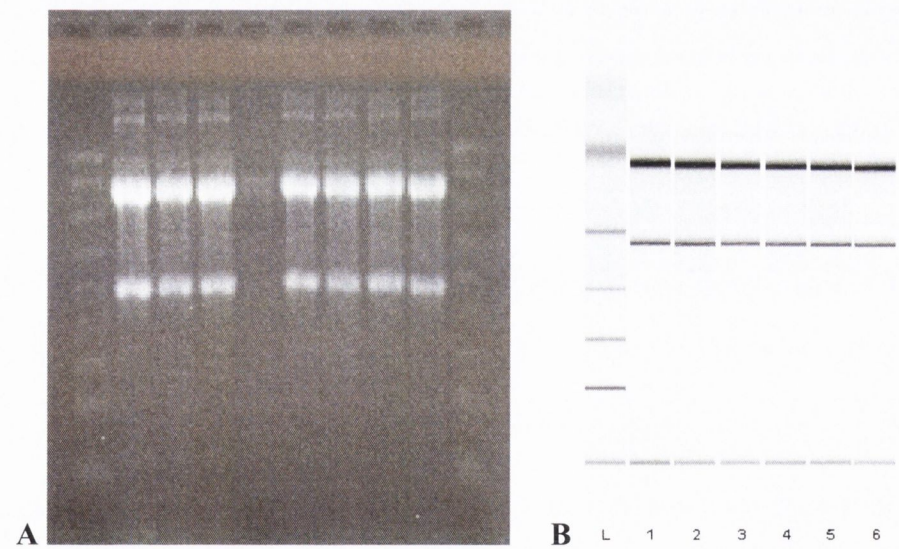

Figure 3.4 RNA quality: A) denaturing formaldehyde agarose gel electrophoresis of RNA; and B) Gel-like data display from Bioanalyzer data.

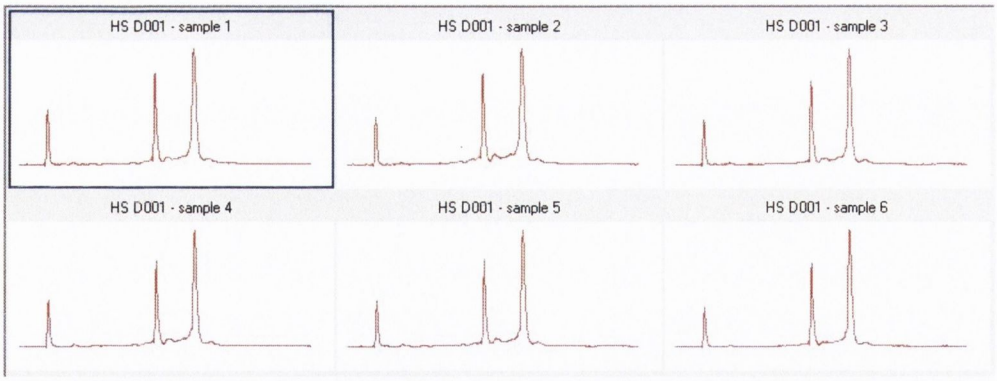

Figure 3.5 Bioanalyser results for RNA samples. The two larger peaks represent the 18S and 28S rRNA bands. RNA degradation would be indicated by a shift in RNA fragment size distribution towards smaller fragments and a decrease in fluorescence signal of the rRNA peaks.

DNA-specific PCR to Control for DNA Contamination of RNA Samples

In order to check for DNA contamination of RNA samples, a small amount of each RNA sample was amplified with primers specific to a section of the MTHFR gene that cross an intron/exon border (Section 2.2.6). This PCR therefore only amplifies genomic DNA, as introns are spliced from RNA during transcription. An example of typical results is shown in Figure 3.6, where a PCR product was seen for amplified non-DNase treated RNA samples (lanes 2 and 3) and control DNA samples (lanes 6 & 7) but not for amplified DNase treated RNA samples (lanes 4 & 5).

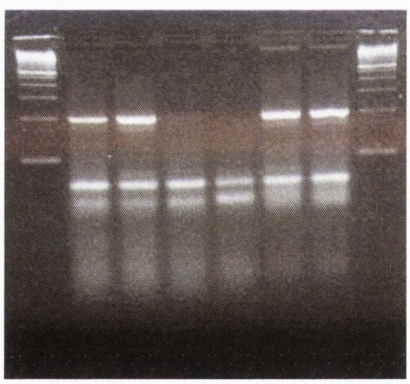

Figure 3.6 PCR products from DNA-specific amplification of a section of the MTHFR gene. Lane 1: 100 bp DNA ladder; lanes 2 & 3: non-DNase treated RNA PCR product; lanes 4 & 5: DNase treated PCR product; lanes 6 & 7: DNA control PCR product; Lane 8: 100 bp DNA ladder. The higher band represents PCR product (198 bp), lower molecular weight bands represent primer dimers.

Spectrophotometry to Determine RNA Quantity and Contamination

A small aliquot of all resuspended RNA samples was diluted in Tris HCl for UV spectrophotometric analysis (Table 3.1). Concentrations and total RNA yield of each sample was calculated from the OD_{260} reading. The resulting concentrations then allowed for resuspension of samples at 1 µg/µl for microarray and TaqMan analysis. For each

sample, the OD_{260}/OD_{280} ratio was above 2.14, meaning no protein contamination of the sample. The OD_{270}/OD_{260} ratio was below 0.8 for all RNA samples, indicating that no phenol contamination of samples had occurred, and the OD_{230}/OD_{260} ratio was below 0.5 for all samples, indicating that there was no contaminating organics in any RNA sample.

Table 3.1 UV spectrophotometer readings, ratios, and calculated initial concentrations and total yield of RNA samples.

	Untreated (1)	LPS (1)	Untreated (2)	PC	L+PC	LPS (2)
OD_{260}	0.165	0.139	0.174	0.202	0.224	0.193
OD_{280}	0.075	0.063	0.079	0.091	0.104	0.087
OD_{230}	0.076	0.062	0.078	0.09	0.103	0.087
OD_{270}	0.128	0.107	0.135	0.155	0.175	0.148
OD_{260}/OD_{280}	2.2	2.2	2.2	2.2	2.15	2.2
OD_{270}/OD_{260}	0.78	0.77	0.78	0.77	0.78	0.77
OD_{230}/OD_{260}	0.46	0.45	0.45	0.45	0.46	0.45
Concentration (µg/µl)	8.8	7.4	9.28	10.77	11.95	10.29
Total RNA yield	140.8	129.73	162.4	177.7	215.1	169.84

cDNA Labelling

The cDNA recovery rate (calculated by dividing the starting amount of total RNA by the amount of resulting labelled cDNA) was found by MWG to be between 1.3 % and 2.4 % for the six RNA samples. cDNA was labelled by direct incorporation, and the label incorporation efficiency varied from 0.4 % to 0.6%. 1 µg of labelled cDNA of both samples to be analysed was hybridised to each array.

3.3.2 Microarray Analysis to Determine Gene Expression Profiles of THP-1 cells

The microarray slide comparing untreated THP-1 cDNA to LPS-treated THP-1 cDNA was designated sl91, that comparing untreated THP-1 cDNA to PC-treated cDNA was designated sl92, and that comparing LPS+PC treated THP-1 cDNA to LPS-treated THP-1 cDNA was designated sl93. The untreated cDNA hybridised to slides sl91 and sl92 was labelled with Cy3, as was the LPS+PC-treated cDNA hybridised to sl93. The LPS-treated cDNA hybridised to slides sl91 and sl93, and the PC-treated cDNA hybridised to sl92, was labelled with Cy5. In each case, both Cy3 and Cy5 labelled samples were hybridised to the same slide (dual hybridisation). General results from the hybridisations are seen in Figures 3.7, 3.8, and 3.9 which show and images of the hybridised microarray slide, histograms of

the normalised ratios for all wells of the arrays, and scatter plots of Cy3 versus Cy5 for all wells of the arrays.

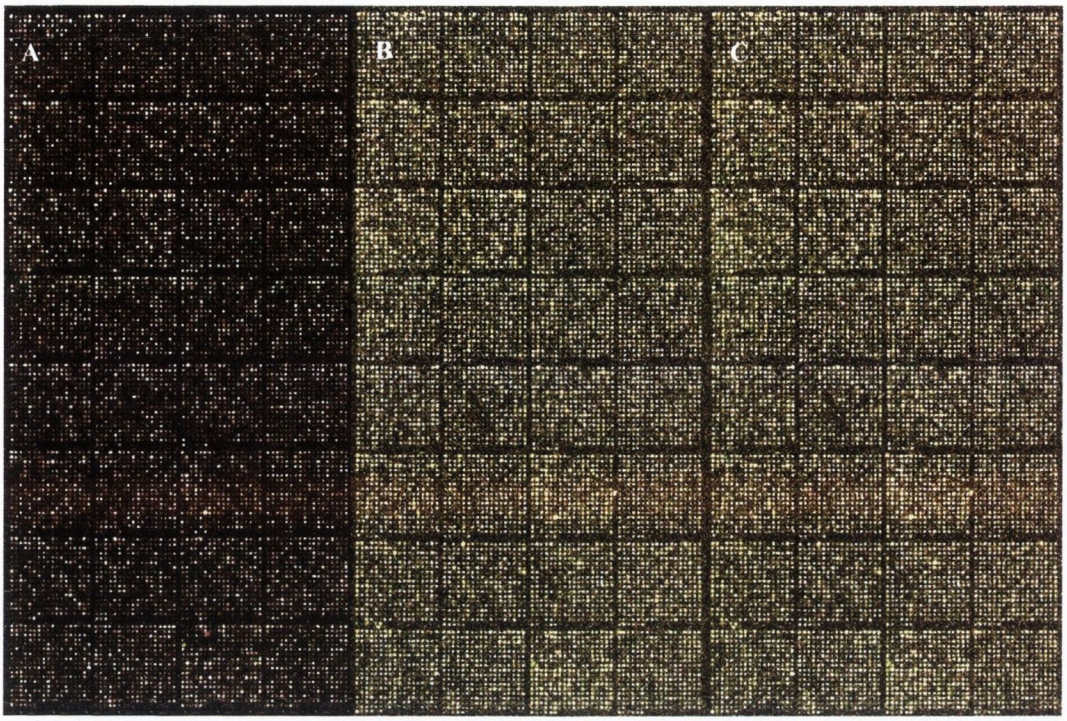

Figure 3.7 Images of microarray slides: (A) sl91 hybridised with Cy3 labelled untreated THP-1 cDNA and Cy5 labelled LPS-treated THP-1 cDNA; (B) sl92 hybridised with Cy3 labelled untreated THP-1 cDNA and Cy5 labelled PC-treated THP-1 cDNA; (C) sl93 microarray slide, hybridised with Cy3 labelled LPS+PC-treated THP-1 cDNA and Cy5 labelled LPS-treated THP-1 cDNA.

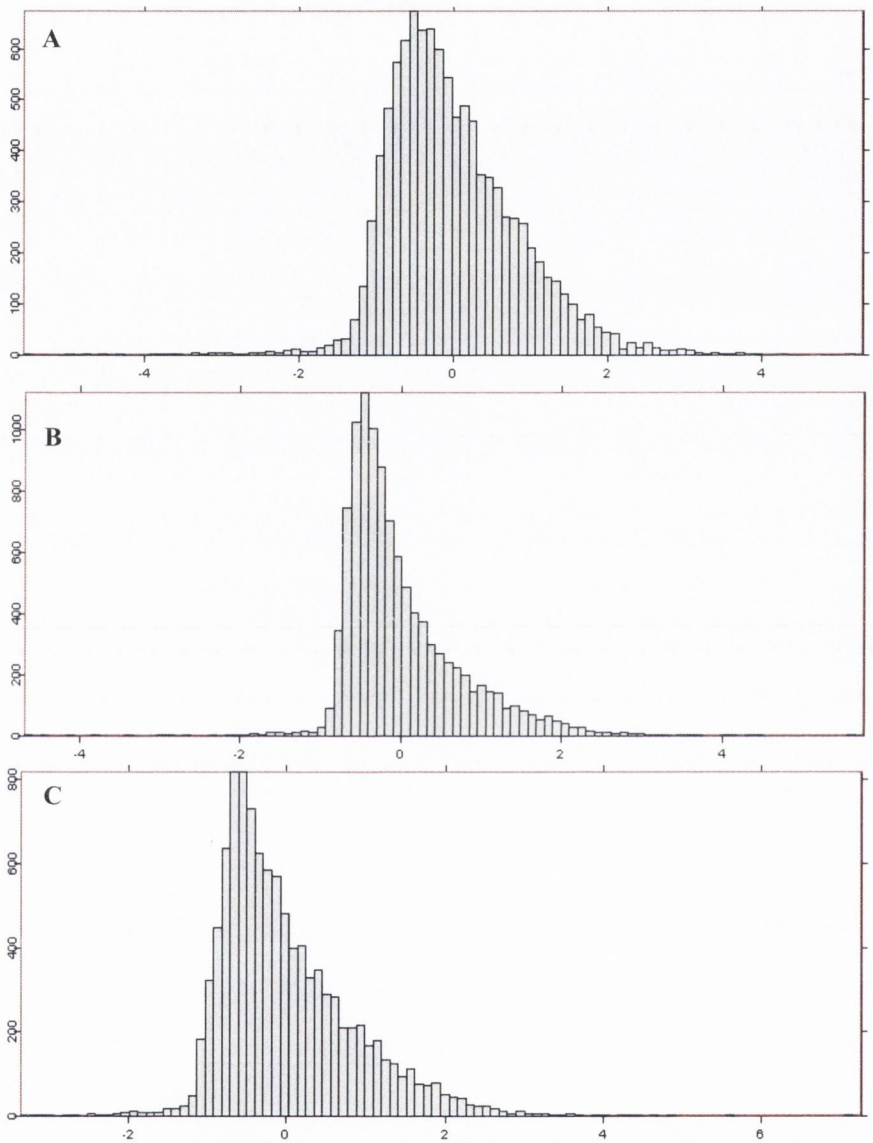

Figure 3.8 Histogram of ratios obtained for: (A) sl91 microarray, (B) sl92 microarray, (C) sl93 microarray. Note that, due to normalisation, the peak of the histograms lie close to zero.

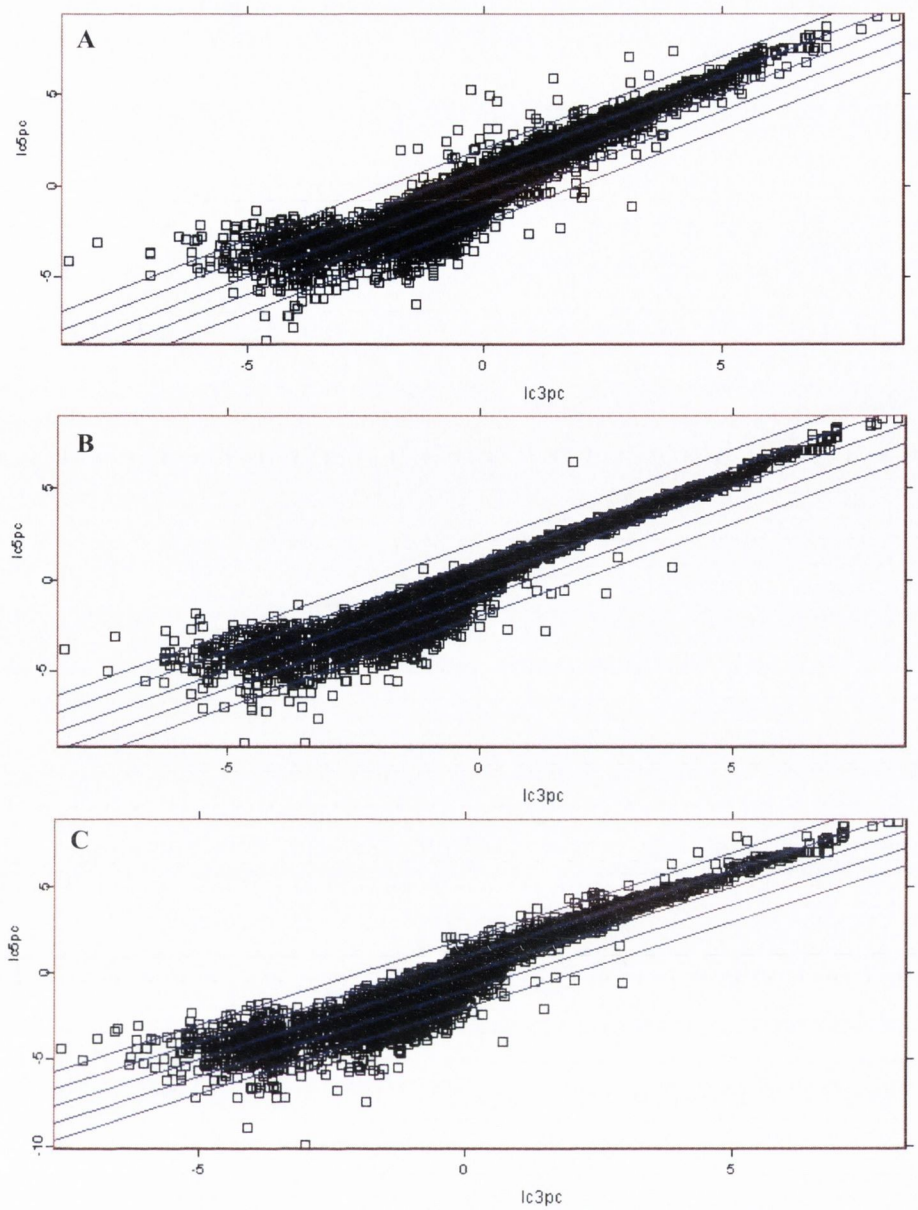

Figure 3.9 Scatter plot of normalised Cy3 versus normalised Cy5 intensity values for each well of: (A) sl91 microarray, (B) sl92 microarray, (C) sl93 microarray. The central blue line indicates a 1:1 ratio of Cy3 and Cy5 intensities, the two lines either side represent two fold up or down regulated wells, and the two outer blue lines represent four fold up or down regulated wells.

3.3.3 Microarray Analysis to Determine Gene Expression Profiles of Untreated and Protein C Treated THP-1 Cells

Figure 3.10 shows the location of the empty wells in the scatter plot of Cy3 *versus* Cy5 intensities for slide sl91, from which it is clear that the background fluorescence recorded in these wells is low. A cut off intensity value for no expression was set twofold above the highest empty well signal. For the sl91 slide, the cut off intensity levels for no expression were therefore set at 290 bytes per pixel for Cy3 intensities and 350 bytes per pixel for Cy5 intensities. Thus any wells with intensity values less than these cut off values were considered to show no gene expression.

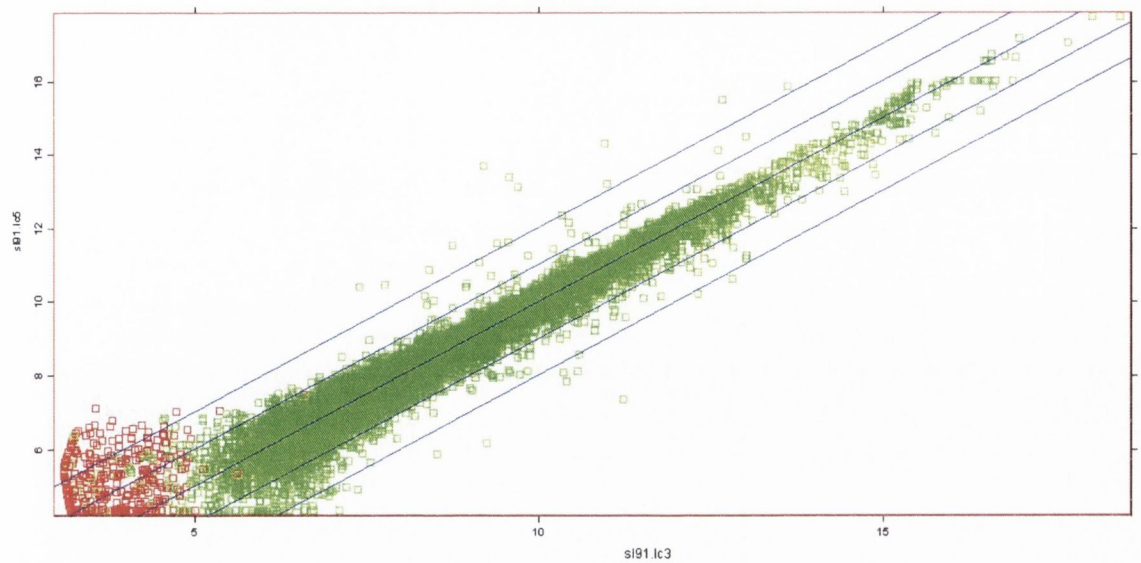

Figure 3.10 Scatter plot of well intensities (from 1 to 17 000 bytes per pixel), Cy3 *versus* Cy5, for the untreated *versus* LPS-treated microarray slide (sl91). Empty wells are shown in red; all other wells are shown in green. The central blue line indicates a 1:1 ratio of Cy3 and Cy5 intensities, the two lines either side represent two fold up or down regulated wells, and the two outer blue lines represent four fold up or down regulated wells.

Results obtained for replicated control wells are shown in Table 3.2. It is clear from these results that all replicates of the same gene give very similar ratios, although the intensity values of replicates vary in some cases. Many of the controls show a 2-fold upregulation in the LPS treated cells compared to the untreated cells, indicating that these genes would not be suitable if used as endogenous housekeeping gene controls. However, β-actin and ARF-1 have ratios close to 1 indicating little variation between the LPS-treated and untreated samples. GAPDH is slightly downregulated by LPS treatment but not to a large extent. These three genes would therefore be considered suitable as endogenous controls in this experiment.

The effect of LPS treatment on THP-1 cells was assessed using transcript profiling with MWG Biotech microarrays. We found that LPS treatment of cells resulted in upregulation of 43 genes by a 3-fold or greater change in expression level (Table 3.3). A further 467 genes were upregulated by a difference in gene expression of between 2- and 2.99-fold, with 46 of these between 2.5- and 2.99-fold (Appendix B (i)).

When investigating the downregulation of gene expression by LPS treatment, 13 wells showed gene expression in both untreated and LPS-treated samples and a gene expression decrease of greater than 2 fold (Table 3.4). Of the 13 wells, only one, representing the thioredoxin interacting protein, had a gene expression difference greater than 3-fold.

Table 3.2 Replicated control wells for slide sl91

Gene Name	GenBank ID	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]
		Untreated (Cy3)	LPS (Cy5)	Untreated (Cy3)	LPS (Cy5)	
Alpha-tubulin	K00558	15957	23883	36.95	65.57	0.56
Alpha-tubulin	K00558	12837	20896	29.73	57.37	0.52
Alpha-tubulin	K00558	18273	30695	42.32	84.27	0.50
Beta-actin	X63432	36203	33021	83.84	90.66	0.92
Beta-actin	X63432	65486	65483	151.65	179.78	0.84
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	X01677	4699	3255	10.88	8.94	1.22
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	X01677	18194	13442	42.13	36.90	1.14
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	X01677	9326	6980	21.60	19.16	1.13
Ribosomal protein L10	L25899	18353	30850	42.50	84.70	0.50
Ribosomal protein L10	L25899	20027	33244	46.38	91.27	0.51
Ribosomal protein L10	L25899	22634	46397	52.42	127.38	0.41
Ribosomal protein L10	L25899	18754	31437	43.43	86.31	0.50
ADP-ribosylation factor 1 (ARF-1)	AF052179	3783	2875	8.76	7.89	1.11
ADP-ribosylation factor 1 (ARF-1)	AF052179	2023	1632	4.68	4.48	1.05
ADP-ribosylation factor 1 (ARF-1)	AF052179	3857	3148	8.93	8.64	1.03
Proteasome (prosome, macropain) subunit, beta type, 3 (PSMB-3)	D26598	2555	4026	5.92	11.05	0.54
Proteasome (prosome, macropain) subunit, beta type, 3 (PSMB-3)	D26598	2690	4979	6.23	13.67	0.46
Heterogeneous nuclear ribonucleoprotein C (HNRPC)	M16342	1630	2649	3.77	7.27	0.52
Heterogeneous nuclear ribonucleoprotein C (HNRPC)	M16342	3825	6751	8.86	18.53	0.48
Heterogeneous nuclear ribonucleoprotein C (HNRPC)	M16342	2197	3843	5.09	10.55	0.48

[a] Calculated by dividing the normalised signal intensity for untreated cells by that for LPS-treated cells.

Table 3.2 continued Replicated control wells for slide sl91

Gene Name	GenBank ID	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]
		Untreated (Cy3)	LPS (Cy5)	Untreated (Cy3)	LPS (Cy5)	
Angio-associated migratory cell protein (AAMP)	M95627	370	185	0.86	0.51	1.69
Angio-associated migratory cell protein (AAMP)	M95627	380	196	0.88	0.54	1.63
Phosphatidylserine synthase 1 (PTDSS-1)	BC004192	1502	2093	3.48	5.75	0.61
Phosphatidylserine synthase 1 (PTDSS-1)	BC004192	1391	1698	3.22	4.66	0.69
Phosphatidylserine synthase 1 (PTDSS-1)	BC004192	846	928	1.96	2.55	0.77
Proteasome (prosome, macropain) subunit, beta type, 2 (PSMB-2)	BC000268	1385	2611	3.21	7.17	0.45
Proteasome (prosome, macropain) subunit, beta type, 2 (PSMB-2)	BC000268	1386	2549	3.21	7.00	0.46
Transcription elongation factor A (SII), 1 (TCEA-1)	X73534	1753	2922	4.06	8.02	0.51
Transcription elongation factor A (SII), 1 (TCEA-1)	X73534	1593	2789	3.69	7.66	0.48
Lysyl-tRNA synthetase nuclear mitochondrial product alternatively spliced isoform	AF285758	1031	1322	2.39	3.63	0.66
G protein pathway suppressor 1 (GPS-1)	NM_004127	721	550	1.67	1.51	1.11
Transcription factor-like 1	BC003151	494	672	1.14	1.85	0.62
Transcription factor-like 1	BC003151	656	1006	1.52	2.76	0.55
Histone H3.3	M77656	18050	24565	41.80	67.44	0.62
Histone H3.3	M77656	12454	21404	28.84	58.76	0.49
Integral membrane protein calnexin ip90 (CANX)	NM_001746	3661	4446	8.48	12.21	0.69
Integral membrane protein calnexin ip90 (CANX)	NM_001746	1776	2581	4.11	7.09	0.58

[a] Calculated by dividing the normalised signal intensity for untreated cells by that for LPS-treated cells.

Table 3.3 Genes that are more than 3-fold upregulated in LPS treated THP1 cells compared to untreated cells

Gene Name	GenBank ID	Function	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]	
			Untreated (Cy3)	LPS (Cy5)	Untreated (Cy3)	LPS (Cy5)	LPS/ Untreated	Untreated /LPS
Interleukin-8 (IL-8)	NM_000584	Chemokine (C-X-C)	356	13245	0.82	36.36	44.11	0.02
Down syndrome critical region protein 1 (DSCR-1)	XM_048638	Calcineurin inhibition	469	10656	1.09	29.26	26.91	0.04
Macrophage inflammatory protein 4 (MIP-4)	NM_002988	Chemokine (C-C)	1175	20327	2.72	55.81	20.51	0.05
Interleukin-1 beta (IL-1β)	NM_000576	Inflammation	522	8894	1.21	24.42	20.19	0.05
Macrophage inflammatory protein 1-beta (MIP-1β)	NM_002984	Chemokine (C-C)	3627	46058	8.40	126.45	15.06	0.07
Fibronectin leucine rich trans-membrane protein 3 (FLRT-3)	NM_013281	Cell adhesion/receptor signalling	130 [b]	1334	0.30	3.66	12.18	0.08
Macrophage inflammatory protein 3-alpha (MIP-3α)	NM_004591	Chemokine (C-C)	297	2931	0.69	8.05	11.70	0.09
Macrophage inflammatory protein 1-alpha (MIP-1α)	NM_002983	Chemokine (C-C)	6841	59590	15.84	163.60	10.33	0.10
Glycoprotein hormones, alpha polypeptide (CG-α)	NM_000735	Common subunit of TSH, FSH, LH, CG glycoprotein hormones	166 [b]	1399	0.38	3.84	10.02	0.10
Serum/glucocorticoid regulated kinase (SGK)	NM_005627	Activation of certain potassium, sodium, and chloride channels	250 [b]	1858	0.58	5.10	8.80	0.11
DNAJ (HSP40) homologue, subfamily A, member 4 (DNAJA4)	NM_018602	Molecular chaperone homologue	1267	9399	2.93	25.81	8.79	0.11

[a] Calculated by dividing the normalised signal intensity for LPS treated cells by that for untreated cells, and for untreated cells by LPS treated cells.
[b] Signal intensity below 'no expression' cut off point (Section 3.3.3), indicating no gene expression and thus unreliable fold difference in gene expression value.
TSH = thyroid stimulating hormone; FSH = follicle stimulating hormone; LH = luteinising hormone; CG = chorionic gonadotrophin.

Table 3.3 continued Genes that are more than 3-fold upregulated in LPS treated THP1 cells compared to untreated cells

Gene Name	GenBank ID	Function	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]	
			Untreated (Cy3)	LPS (Cy5)	Untreated (Cy3)	LPS (Cy5)	LPS/ Untreated	Untreated /LPS
CD38 antigen (p45) (CD38)	NM_001775	Metabolism of cADPR. Roles in cell proliferation & neutrophil chemotaxis	465	3096	1.08	8.50	7.90	0.13
Spermidine/spermine N1-acetyltransferase (SAT)	NM_002970	Cell growth, possible involvement in monocyte activation	857	5206	1.98	14.29	7.20	0.14
Nuclear factor, interleukin 3 regulated (NFIL-3)	NM_005384	T cell IL-3 expression regulation, possible anti-apoptotic role	520	3156	1.20	8.66	7.20	0.14
CD83 antigen (CD83)	NM_004233	Dendritic cell marker	408	2125	0.94	5.83	6.18	0.16
Kinesin family member 4A (KIF-4A)	NM_012310	Transport	928	4498	2.15	12.35	5.75	0.17
Tumor necrosis factor alpha (TNF-α)	NM_000594	Inflammation	3696	17812	8.56	48.90	5.71	0.18
Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha (NFκBIA)	NM_020529	NFκB inhibition	4812	22750	11.14	62.46	5.61	0.18
Microrchidia (mouse) homologue (MORC)	NM_014429	Testis-specific, possible apoptosis induction	257 [b]	1127	0.60	3.09	5.20	0.19
Dual specificity phosphatase 1 (DUSP-1)	NM_004417	Response to environmental stress, regulation of cell proliferation	967	3749	2.24	10.29	4.60	0.22
Coagulation factor III precursor (tissue factor, TF) (FIII)	NM_001993	Coagulation	380	1442	0.88	3.96	4.50	0.22
Nuclear factor of kappa light polypeptide gene enhancer in B cells 1 (p105) (NFκB-1)	NM_003998	Inflammatory gene transcription activation	654	2448	1.52	6.72	4.44	0.23

[a] Calculated by dividing the normalised signal intensity for LPS treated cells by that for untreated cells, and for untreated cells by LPS treated cells.
[b] Signal intensity below 'no expression' cut off point (Section 3.3.3), indicating no gene expression and thus unreliable fold difference in gene expression value.
cADPR = cyclic ADP-ribose.

Table 3.3 continued Genes that are more than 3-fold upregulated in LPS treated THP1 cells compared to untreated cells

Gene Name	GenBank ID	Function	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]	
			Untreated (Cy3)	LPS (Cy5)	Untreated (Cy3)	LPS (Cy5)	LPS/ Untreated	Untreated /LPS
Zinc finger protein 133 (ZNF-133)	NM_003434	Regulation of cell proliferation and differentiation	285 [b]	1050	0.66	2.88	4.36	0.23
BTG family, member 2 (BTG-2)	NM_006763	Cell cycle regulation	1685	6071	3.90	16.67	4.27	0.23
Tumor necrosis factor alpha-induced protein 6 (TNF-αIP6)	NM_007115	Anti-inflammatory, activates inter-α-inhibitor	378	1350	0.88	3.71	4.23	0.24
Connective tissue growth factor (CTGF)	NM_001901	Role in connective tissue formation and during wound healing	272 [b]	963	0.63	2.64	4.20	0.24
Receptor-interacting serine-threonine kinase 2 (RIPK-2)	NM_003821	Signal transducer for both innate and adaptive immune responses	476	1675	1.10	4.60	4.17	0.24
GRO-1 oncogene (melanoma growth stimulating activity, alpha) (GRO-1)	NM_001511	Chemokine (C-X-C)	1856	6420	4.30	17.63	4.10	0.24
Nuclear factor (erythroid-derived 2)-like 2 (NFE-2L2)	NM_006164	Transcription factor: glutathione synthesis regulation and cellular detoxification	1636	5658	3.79	15.53	4.10	0.24
Hypothetical protein	XM_071456	Unknown	836	2738	1.94	7.52	3.88	0.26
Regulated upon activation, normally T-expressed and presumably secreted (RANTES)	NM_002985	Chemokine (C-C)	1606	5249	3.72	14.41	3.87	0.26
Superoxide dismutase 2, mitochondrial (SOD-2)	NM_000636	Oxygen-free radical degradation	1130	3516	2.62	9.65	3.69	0.27
Vimentin (VIM)	NM_003380	Cytoskeletal element	2864	8390	6.63	23.03	3.47	0.29

[a] Calculated by dividing the normalised signal intensity for LPS treated cells by that for untreated cells, and for untreated cells by LPS treated cells.
[b] Signal intensity below 'no expression' cut off point (Section 3.3.3), indicating no gene expression and thus unreliable fold difference in gene expression value.

Table 3.3 continued Genes that are more than 3-fold upregulated in LPS treated THP1 cells compared to untreated cells

Gene Name	GenBank ID	Function	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]	
			Untreated (Cy3)	LPS (Cy5)	Untreated (Cy3)	LPS (Cy5)	LPS/ Untreated	Untreated /LPS
Growth arrest and dna-damage-inducible, alpha (GADD-45α)	NM_001924	Activation of p38/JNK pathway in response to environmental stress	601	1692	1.39	4.65	3.34	0.30
Hypothetical protein	NM_024972	Unknown	1610	4422	3.73	12.14	3.26	0.31
Annexin A1 (ANX-A1)	NM_000700	Anti-inflammatory, inhibition of phospholipase A2	3953	10824	9.15	29.72	3.25	0.31
Interleukin-23, alpha subunit p19 (IL-23α)	NM_016584	Stimulation of IFN-γ production	181 [b]	493	0.42	1.35	3.22	0.31
V-fos FBJ murine osteosarcoma viral oncogene homologue (FOS)	NM_005252	Dimerisation with JUN family members to form AP-1 transcription factor	1162	3150	2.69	8.65	3.21	0.31
Down syndrome critical region protein 1 (DSCR-1)	NM_004414	Calcineurin inhibition	3188	8534	7.38	23.43	3.17	0.32
Ribosomal protein S13 (RPS13)	NM_001017	Component of 40S ribosomal subunit	23657	61439	54.78	168.68	3.08	0.32
Regulator of G-protein signalling 2, 24kd (RGS-2)	NM_002923	Involvement in dysregulated cell growth	543	1402	1.26	3.85	3.06	0.33
Cholinergic receptor, nicotinic, alpha polypeptide 7 (CHRNA-7)	NM_000746	Ligand gated ion channel, mediation of fast signal transmission at synapses	615	1581	1.42	4.34	3.05	0.33
Ribosomal protein L37 (RPL37)	NM_000997	Component of 60S ribosomal subunit	25182	63703	58.32	174.89	3.00	0.33

[a] Calculated by dividing the normalised signal intensity for LPS treated cells by that for untreated cells, and for untreated cells by LPS treated cells.
[b] Signal intensity below 'no expression' cut off point (Section 3.3.3), indicating no gene expression and thus unreliable fold difference in gene expression value.

Table 3.4 Genes that are more than 2-fold downregulated in LPS-treated THP1 cells compared to untreated cells

Gene Name	GenBank ID	Function	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]	
			Untreated (Cy3)	LPS (Cy5)	Untreated (Cy3)	LPS (Cy5)	LPS/ Untreated	Untreated /LPS
Thioredoxin interacting protein (TXNIP)	NM_006472	Regulation of cellular redox state	1657	377	3.84	1.04	0.27	3.70
Peroxisomal long-chain acyl-coA thioesterase (ZAP-128)	NM_006821	Regulation of peroxisomal lipid metabolism	5409	1554	12.53	4.27	0.34	2.94
Toll-like receptor 9 (TLR-9)	NM_017442	Mediation of immune response to unmethylated CpG dinucleotides (i.e. bacterial DNA)	4013	1180	9.29	3.24	0.35	2.87
Granulin (GRN)	NM_002087	Wound related growth factor	1654	591	3.83	1.62	0.42	2.36
Moesin (MSN)	NM_002444	Cross linker between plasma membranes and actin-based cytoskeletons	2141	765	4.96	2.10	0.42	2.36
Protease, serine, 15 (PRSS-15)	NM_004793	Regulation of DNA replication and transcription in the mitochondria	1009	373	2.34	1.02	0.44	2.28
Valosin-containing protein (VCP)	AF313485	Nuclear ATPase, membrane fusion, ubiquitin-dependent protein degradation	916	357	2.12	0.98	0.46	2.16
Hypothetical protein	NM_017974	Unknown	5876	2301	13.61	6.32	0.46	2.15
Hypothetical protein	NM_017763	Unknown	929	378	2.15	1.04	0.48	2.07
Hypothetical protein	XM_040853	Unknown	2786	1136	6.45	3.12	0.48	2.07
Hypothetical protein	NM_021215	Unknown	5934	2434	13.74	6.68	0.49	2.06
Eukaryotic translation initiation factor 4h, isoform 1 (WBSCR-1)	NM_022170	Translation initiation factor	1873	770	4.34	2.11	0.49	2.05
Hypothetical protein	NM_018150	Unknown	1229	506	2.85	1.39	0.49	2.05

[a] Calculated by dividing the normalised signal intensity for LPS treated cells by that for untreated cells, and for untreated cells by LPS treated cells.

However, many wells showed some expression in the untreated sample but no expression in the LPS-treated sample (intensity below the cut off point). This does not allow for an accurate estimate of the fold downregulation, as the low intensity values seen when a gene is not expressed in a sample, caused by background fluorescence, can vary between wells thus affecting the ratio calculation. In order to get a rough estimate of the decrease in gene expression seen in these wells, we therefore set all wells with Cy5 (LPS sample) intensity value at or below 350 bytes per pixel to 350 bytes per pixel, the equivalent of a normalised value (sl91.Ic5pc) of 0.96. The normalised ratio was then calculated using this revised Cy5 value and the Cy3 (untreated) sample normalised intensity. Table 3.5 shows wells with a 2-fold or greater fold difference in gene expression using this method of setting the intensity of Cy5 wells with no expression to 350 bytes per pixel. Wells with a fold difference between 1.99 and 1.5 are shown in Appendix B (ii).

3.3.4 Microarray Analysis to Determine Gene Expression Profiles of Untreated and Protein C Treated THP-1 Cells

The intensity value of empty wells in the sl92 microarray ranged from 1.7 to 147 bytes per pixel for Cy3, and from 1 to 190 bytes per pixel for Cy5. A cut off intensity value for no expression was set twofold above the highest empty well signal. The cut off intensity levels for no expression were therefore set at 295 bytes per pixel for Cy3 intensities and 380 bytes per pixel for Cy5 intensities. Thus any wells with intensity values less than these cut off values were considered to show no gene expression.

Results obtained for replicated control wells are shown in Table 3.6. In all cases, ratios of replicates were very similar. Genes with ratios closest to 1 included GAPDH, ARF-1, AAMP, and PTDSS-1 which would therefore be suitable as endogenous controls in this experiment.

The effect of PC treatment on THP-1 cells was assessed using transcript profiling. PC treatment of cells resulted in upregulation of 1 gene, encoding INE-1, by a 3-fold or greater change in expression level (Table 3.7). A second gene was found to have a fold gene expression difference of 3.15, however, this gene was not expressed in the untreated cDNA sample (Cy3-labelled), and therefore this Cy3 intensity value may result in an unreliable ratio of gene expression difference. If the Cy3 cut-off intensity value of 295 bytes per pixel (corresponding to a normalised intensity of 0.61) (see paragraph 1 of this section) is used

Table 3.5 Genes that are expressed in untreated cells but show no expression in LPS-treated cells

Gene Name	GenBank ID	Function	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]
			Untreated (Cy3)	LPS (Cy5)	Untreated (Cy3)	LPS (Cy5)	
PRO-0659 protein	NM_014138	Unknown	3727	163 [b]	8.63	0.45	8.99
Hypothetical protein	NM_017984	Unknown	1867	275 [b]	4.32	0.75	4.50
Hypothetical protein	NM_024956	Unknown	1816	228 [b]	4.21	0.63	4.38
Neural cell adhesion molecule 1 (NCAM-1)	NM_000615	Cell-cell and cell-matrix adhesion	1637	268 [b]	3.79	0.74	3.95
MAS1 oncogene (MAS-1)	NM_002377	Receptor, may modulate cell growth pathway	1312	71 [b]	3.04	0.19	3.17
Xeroderma pigmentosum, complementation group C (XPC)	NM_004628	DNA repair	1132	275 [b]	2.62	0.75	2.73
PM-5 protein	NM_014287	Connective tissue component	1071	260 [b]	2.48	0.71	2.58
Diaphanous 1 (DIAPH-1)	NM_005219	Regulation of actin polymerisation	954	272 [b]	2.21	0.75	2.30
GLUT4 enhancer factor (GEF)	NM_020062	Transcriptional regulation of GLUT4 gene (insulin-responsive glucose transporter)	928	282 [b]	2.15	0.77	2.24
Cathepsin D (lysosomal aspartyl protease) (CTSD)	NM_001909	Protease, breast cancer involvement	902	248 [b]	2.09	0.68	2.18
Quinolinate phosphoribosyltransferase (QPRT)	NM_014298	Catabolism of quinolate, an endogenous excitotoxin to neurons	854	240 [b]	1.98	0.66	2.06
cAMP-regulated guanine nucleotide exchange factor II (cAMP-GEFII)	NM_007023	Involvement in cAMP signalling independent of PKA, activation of Rap1 and 2	829	58 [b]	1.92	0.16	2.00
Integrin alpha 5 precursor (ITG-A5)	NM_002205	Part of the fibronectin receptor, an integrin, involved in adhesion and cell surface mediated signalling	827	251 [b]	1.92	0.69	2.00

[a] Calculated by dividing the normalised signal intensity for untreated cells by 0.96. This was double the highest normalised intensity value displayed by an empty well and was taken as the cut-off point for no expression in the LPS-treated sample (0.96 corresponds to an intensity value of 350 bytes per pixel).

[b] Signal intensity below 'no expression' cut off point (Section 3.3.3), indicating no gene expression. This value can not therefore be used for calculation of gene expression ratio as results obtained would be unreliable due to slight fluctuations in background fluorescence between wells.

Table 3.6 Replicated control wells for slide sl92

Gene Name	GenBank ID	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]
		Untreated (Cy3)	PC (Cy5)	Untreated (Cy3)	PC (Cy5)	
Alpha-tubulin	K00558	18127	22451	37.22	43.96	0.85
Alpha-tubulin	K00558	18798	29801	38.60	58.35	0.66
Alpha-tubulin	K00558	21344	33982	43.82	66.54	0.66
Beta-actin	X63432	65220	107084	133.91	209.67	0.64
Beta-actin	X63432	112022	176036	230.00	344.68	0.67
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	X01677	10513	11889	21.58	23.28	0.93
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	X01677	21766	23716	44.69	46.44	0.96
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	X01677	10763	12590	22.10	24.65	0.90
Ribosomal protein L10	L25899	23723	42317	48.71	82.86	0.59
Ribosomal protein L10	L25899	22506	39361	46.21	77.07	0.60
Ribosomal protein L10	L25899	19609	30469	40.26	59.66	0.67
Ribosomal protein L10	L25899	23457	37624	48.16	73.67	0.65
ADP-ribosylation factor 1 (ARF-1)	AF052179	4257	5437	8.74	10.65	0.82
ADP-ribosylation factor 1 (ARF-1)	AF052179	2597	3362	5.33	6.58	0.81
ADP-ribosylation factor 1 (ARF-1)	AF052179	4131	5867	8.48	11.49	0.74
Proteasome (prosome, macropain) subunit, beta type, 3 (PSMB-3)	D26598	3828	5627	7.86	11.02	0.71
Proteasome (prosome, macropain) subunit, beta type, 3 (PSMB-3)	D26598	5474	9901	11.24	19.39	0.58
Heterogeneous nuclear ribonucleoprotein C (HNRPC)	M16342	6400	10373	13.14	20.31	0.65
Heterogeneous nuclear ribonucleoprotein C (HNRPC)	M16342	9736	17661	19.99	34.58	0.58
Heterogeneous nuclear ribonucleoprotein C (HNRPC)	M16342	4322	7546	8.87	14.78	0.60

[a] Calculated by dividing the normalised signal intensity for untreated cells by that for PC-treated cells.

Table 3.6 continued Replicated control wells for slide sl92

Gene Name	GenBank ID	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]
		Untreated (Cy3)	PC (Cy5)	Untreated (Cy3)	PC (Cy5)	
Angio-associated migratory cell protein (AAMP)	M95627	523	492	1.07	0.96	1.11
Angio-associated migratory cell protein (AAMP)	M95627	483	540	0.99	1.06	0.94
Phosphatidylserine synthase 1 (PTDSS-1)	BC004192	3176	4384	6.52	8.58	0.76
Phosphatidylserine synthase 1 (PTDSS-1)	BC004192	2764	3848	5.68	7.53	0.75
Phosphatidylserine synthase 1 (PTDSS-1)	BC004192	3300	4265	6.77	8.35	0.81
Proteasome (prosome, macropain) subunit, beta type, 2 (PSMB-2)	BC000268	2056	3632	4.22	7.11	0.59
Proteasome (prosome, macropain) subunit, beta type, 2 (PSMB-2)	BC000268	2480	4471	5.09	8.75	0.58
Transcription elongation factor A (SII), 1 (TCEA-1)	X73534	2398	3846	4.92	7.53	0.65
Transcription elongation factor A (SII), 1 (TCEA-1)	X73534	2463	4240	5.06	8.30	0.61
Lysyl-tRNA synthetase nuclear mitochondrial product alternatively spliced isoform	AF285758	3665	5593	7.53	10.95	0.69
G protein pathway suppressor 1 (GPS-1)	NM_004127	528	650	1.08	1.27	0.85
Transcription factor-like 1	BC003151	1239	1993	2.54	3.90	0.65
Transcription factor-like 1	BC003151	837	1307	1.72	2.56	0.67
Histone H3.3	M77656	23434	37322	48.11	73.08	0.66
Histone H3.3	M77656	20313	33919	41.71	66.41	0.63
Integral membrane protein calnexin ip90 (CANX)	NM_001746	9454	15794	19.41	30.93	0.63
Integral membrane protein calnexin ip90 (CANX)	NM_001746	9291	16205	19.08	31.73	0.60

[a] Calculated by dividing the normalised signal intensity for untreated cells by that for PC-treated cells.

Table 3.7 Genes that are more than 2-fold upregulated in PC treated THP1 cells compared to untreated cells

Gene Name	GenBank ID	Function	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]	
			Untreated (Cy3)	PC (Cy5)	Untreated (Cy3)	PC (Cy5)	PC/ Untreated	Untreated /PC
INE-1	Y10696	Expressed from both X chromosomes in the female, function unknown	1745	43729	3.58	85.62	23.90	0.04
Chromosome 11 open reading frame 1 (C11orf1)	NM_022761	Nuclear protein, function unknown	230 [b]	759	0.47	1.49	3.15	0.32
PRO-0644 protein	XM_037037	Unknown	47352	117869	97.22	230.79	2.37	0.42
Spermatogenesis associated 1 (SPATA-1)	NM_022354	Unknown	345	836	0.71	1.64	2.31	0.43
Eukaryotic translation elongation factor 1 alpha-2 (EEF1A2)	NM_001958	Subunit of elongation factor-1 complex which delivers aminoacyl tRNAs to the ribosome, oncogenic properties	65501	153357	134.48	300.27	2.23	0.45
Clone FLB3816	NM_016415	Unknown	64834	150538	133.12	294.75	2.21	0.45
Nuclear ubiquitous casein kinase and cyclin-dependent kinase substrate (NUCKS gene)	AJ012584	DNA binding protein, possible involvement in cell proliferation	1472	3314	3.02	6.49	2.15	0.47
Hypothetical protein	NM_024972	Unknown	1255	2802	2.58	5.49	2.13	0.47
Ribosomal protein L23A (RPL23A)	NM_000984	Component of ribosomal 60S subunit	25165	54813	51.67	107.32	2.08	0.48
Ribosomal protein P0 (RPLP0)	NM_053275	Component of ribosomal 60S subunit	65488	142160	134.46	278.35	2.07	0.48
Integrin, beta-like 1 (with EGF-like repeat domains) (ITGBL1)	NM_004791	Possible cell adhesion and recognition activities	2468	5261	5.07	10.30	2.03	0.49

[a] Calculated by dividing the normalised signal intensity for PC-treated cells by that for untreated cells, and for untreated cells by PC-treated cells.
[b] Signal intensity below 'no expression' cut off point (Section 3.3.4), indicating no gene expression and thus unreliable fold difference in gene expression value.

instead of the apparent intensity of 230 bytes per pixel, the ratio falls to 2.15. A further 9 genes were upregulated by a difference in gene expression of between 2- and 3-fold (Table 3.7).

When investigating the downregulation of gene expression by PC treatment, 3 wells showed gene expression in both untreated and PC-treated samples and a gene expression decrease of greater than 2 fold (Table 3.8). Of these 3 wells, only one, representing endomucin-2, had a gene expression difference greater than 3-fold. Many wells showed some expression in the untreated sample but no expression in the PC-treated sample (intensity below the cut off point). As discussed in the section above, this does not allow for an accurate estimate of the fold downregulation. Therefore, in order to get a rough estimate of the decrease in gene expression seen in these wells, we set all wells with Cy5 (PC sample) intensity value at or below 380 bytes per pixel to 380 bytes per pixel, the equivalent of a normalised value (sl91.Ic5pc) of 0.74 and the normalised ratio was calculated using this revised Cy5 value with the Cy3 (untreated) sample normalised intensity. Table 3.9 shows wells with a 2-fold or greater fold difference in gene expression using this method. Wells with a fold difference between 1.99 and 1.5 are shown in Appendix B (iii).

3.3.5 Microarray Analysis to Determine Gene Expression Profiles of LPS-treated and LPS + Protein C Treated THP-1 Cells

Intensity values obtained for empty wells in the sl93 microarray ranged from 1 to 142 bytes per pixel for Cy3, and 1 to 156 bytes per pixel for Cy5. Thus a cut off point of 284 bytes per pixel and 312 bytes per pixel for Cy3 and Cy5 respectively was used to determine wells with no gene expression. Any wells with intensity values below these amounts were considered to show no expression of the gene represented by them.

Data from replicate wells in array sl93 are shown in Table 3.10. As seen in the other two slides, most replicates had similar ratios, although intensities between replicate wells varied in some cases. In this slide, there was a greater difference between the LPS+PC- and LPS-treated samples than was seen for the untreated *versus* LPS-treated and untreated *versus* PC-treated slides, with higher gene expression generally seen in the LPS sample compared to the LPS+PC sample. Wells with a gene expression ratio closest to 1 included those representing alpha tubulin, GAPDH, and PTDSS-1.

Table 3.8 Genes that are more than 2-fold downregulated in PC treated THP1 cells compared to untreated cells

Gene Name	GenBank ID	Function	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]	
			Untreated (Cy3)	PC (Cy5)	Untreated (Cy3)	PC (Cy5)	PC/ Untreated	Untreated /PC
Endomucin-2	NM_016242	Potential role in tumour angiogenesis	6865.61	778.86	14.10	1.53	0.11	9.24
Toll-like receptor 9 (TLR-9)	NM_017442	Mediation of immune response to unmethylated CpG dinucleotides (i.e. bacterial DNA)	3285.85	1176.40	6.75	2.30	0.34	2.93
Thyroid hormone receptor interactor 12 (TRIP-12)	NM_004238	Hormone dependent interaction with thryoid hormone receptors	936.32	434.63	1.92	0.85	0.44	2.26

[a] Calculated by dividing the normalised signal intensity for PC-treated cells by that for untreated cells, and for untreated cells by PC-treated cells.

Table 3.9 Genes that are expressed in untreated cells but show no expression in PC-treated cells

Gene Name	GenBank ID	Function	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]
			Untreated (Cy3)	PC (Cy5)	Untreated (Cy3)	PC (Cy5)	
PRO-0659 protein	NM_014138	Unknown	2752	299 [b]	5.65	0.59	7.63
Hypothetical protein	NM_017984	Unknown	1578	326 [b]	3.24	0.64	4.38
Neural cell adhesion molecule 1 (NCAM-1)	NM_000615	Cell-cell and cell-matrix adhesion	1306	296 [b]	2.68	0.58	3.62
MAS-1 oncogene	NM_002377	Receptor, may modulate cell growth pathway	1198	73 [b]	2.46	0.14	3.32
Xeroderma pigmentosum, complementation group C (XPC)	NM_004628	DNA repair	1080	334 [b]	2.22	0.65	3.00
cAMP-regulated guanine nucleotide exchange factor II (cAMP-GEFII)	NM_007023	Involvement in cAMP signalling independent of PKA, activation of Rap1 and 2	729	75 [b]	1.50	0.15	2.02

[a] Calculated by dividing the normalised signal intensity for untreated cells by 0.74. This was double the highest normalised intensity value displayed by an empty well and was taken as the cut-off point for no expression in the PC-treated sample (0.74 corresponds to an intensity value of 380 bytes per pixel).

[b] Signal intensity below 'no expression' cut off point (Section 3.3.4), indicating no gene expression. This value can not therefore be used for calculation of gene expression ratio as results obtained would be unreliable due to slight fluctuations in background fluorescence between wells.

Table 3.10 Replicated control wells for slide sl93

Gene Name	GenBank ID	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]
		LPS + PC (Cy3)	LPS (Cy5)	LPS + PC (Cy3)	LPS (Cy5)	
Alpha-tubulin	K00558	4575	6862	9.60	13.22	0.73
Alpha-tubulin	K00558	20604	33036	43.25	63.63	0.68
Alpha-tubulin	K00558	21765	36602	45.69	70.50	0.65
Beta-actin	X63432	40127	122428	84.23	235.81	0.36
Beta-actin	X63432	64881	187018	136.20	360.21	0.38
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	X01677	11474	20219	24.09	38.94	0.62
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	X01677	4953	8475	10.40	16.32	0.64
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	X01677	7670	11270	16.10	21.71	0.74
Ribosomal protein L10	L25899	23176	43101	48.65	83.02	0.59
Ribosomal protein L10	L25899	20484	37184	43.00	71.62	0.60
Ribosomal protein L10	L25899	22165	43432	46.53	83.65	0.56
Ribosomal protein L10	L25899	23178	44727	48.66	86.15	0.56
ADP-ribosylation factor 1 (ARF-1)	AF052179	3343	6690	7.02	12.89	0.54
ADP-ribosylation factor 1 (ARF-1)	AF052179	2574	4977	5.40	9.59	0.56
ADP-ribosylation factor 1 (ARF-1)	AF052179	1792	3651	3.76	7.03	0.54
Proteasome (prosome, macropain) subunit, beta type, 3 (PSMB-3)	D26598	4123	6900	8.66	13.29	0.65
Proteasome (prosome, macropain) subunit, beta type, 3 (PSMB-3)	D26598	2409	4441	5.06	8.55	0.59
Heterogeneous nuclear ribonucleoprotein C (HNRPC)	M16342	7534	14904	15.82	28.71	0.55
Heterogeneous nuclear ribonucleoprotein C (HNRPC)	M16342	1896	3520	3.98	6.78	0.59
Heterogeneous nuclear ribonucleoprotein C (HNRPC)	M16342	7422	15470	15.58	29.80	0.52

[a] Calculated by dividing the normalised signal intensity for LPS+PC-treated cells by that for LPS-treated cells.

Table 3.10 continued Replicated control wells for slide sl93

Gene Name	GenBank ID	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]
		LPS + PC (Cy3)	LPS (Cy5)	LPS + PC (Cy3)	LPS (Cy5)	
Angio-associated migratory cell protein (AAMP)	M95627	318	275	0.67	0.53	1.26
Angio-associated migratory cell protein (AAMP)	M95627	418	577	0.88	1.11	0.79
Phosphatidylserine synthase 1 (PTDSS-1)	BC004192	3144	4855	6.60	9.35	0.71
Phosphatidylserine synthase 1 (PTDSS-1)	BC004192	2755	4261	5.78	8.21	0.70
Phosphatidylserine synthase 1 (PTDSS-1)	BC004192	3108	4987	6.52	9.61	0.68
Proteasome (prosome, macropain) subunit, beta type, 2 (PSMB-2)	BC000268	2842	5883	5.97	11.33	0.53
Proteasome (prosome, macropain) subunit, beta type, 2 (PSMB-2)	BC000268	2244	4510	4.71	8.69	0.54
Transcription elongation factor A (SII), 1 (TCEA-1)	X73534	639	885	1.34	1.70	0.79
Transcription elongation factor A (SII), 1 (TCEA-1)	X73534	2288	4322	4.80	8.32	0.58
Lysyl-tRNA synthetase nuclear mitochondrial product alternatively spliced isoform	AF285758	1138	2141	2.39	4.12	0.58
G protein pathway suppressor 1 (GPS-1)	NM_004127	733	1016	1.54	1.96	0.79
Transcription factor-like 1	BC003151	396	707	0.83	1.36	0.61
Transcription factor-like 1	BC003151	882	1457	1.85	2.81	0.66
Histone H3.3	M77656	19405	39708	40.73	76.48	0.53
Histone H3.3	M77656	17863	34994	37.50	67.40	0.56
Integral membrane protein calnexin ip90 (CANX)	NM_001746	4309	8240	9.04	15.87	0.57
Integral membrane protein calnexin ip90 (CANX)	NM_001746	4191	8182	8.80	15.76	0.56

[a] Calculated by dividing the normalised signal intensity for LPS+PC-treated cells by that for LPS-treated cells.

The effect of PC treatment on LPS-stimulated THP-1 cells revealed 33 genes 3-fold or more upregulated in LPS-treated cells compared to LPS + PC-treated cells (Table 3.11). A further 321 genes were upregulated by between 2.99- and 2-fold, with 25 of these between 2.5- and 2.99-fold (Appendix B (iv)).

When investigating the downregulation of gene expression by LPS treatment compared to LPS + PC treatment, eight wells showed gene expression in both LPS + PC-treated and LPS-treated samples and a gene expression decrease of greater than 2 fold (Table 3.12). Of the eight wells, four had a gene expression difference greater than 3-fold. Many wells showed some expression in the LPS + PC-treated sample but no expression in the LPS-treated sample (intensity below the cut off point), precluding an accurate estimate of the fold downregulation due to the variation in low intensity values obtained for the LPS sample. In order to get a rough estimate of the decrease in gene expression seen in these wells, we set all wells with Cy5 (LPS sample) intensity value at or below 312 bytes per pixel to 312 bytes per pixel, the equivalent of a normalised value (sl93.Ic5pc) of 0.6. The normalised ratio was then calculated using this revised Cy5 value and the unchanged Cy3 (LPS + PC) sample normalised intensity. Table 3.13 shows wells with a 2-fold or greater difference in gene expression using this method. The only well with a resultant gene expression difference greater than 3 fold represented the MAS-1 oncogene. Wells with a fold difference between 1.99 and 1.5 are shown in Appendix B (v).

3.3.6 TaqMan Analysis of Relative Cytokine Gene Expression After LPS and/or APC or PC Treatment

Determination of Relative Cytokine Gene Expression in LPS and/or PC Treated THP-1 Cells by Real Time PCR

cDNA was made using RNA produced for microarray analysis (Section 3.2.1). Each well of the cytokine plate was loaded with reaction mix containing 533 ng of cDNA. Relative gene expression of cytokines is shown in Figure 3.11. There was no expression of IL-2, IL-4, IL-5, IL-12 (p35 and p40), IL-15, or IFN-γ in any sample. Gene expression of IL-1α, IL-1β, IL-8, IL-10, and TNF-α was increased in LPS treated cells, decreased in LPS + PC treated cells, and low in untreated and PC-treated cells (Figure 3.11). The logarithmic amplification plot of ΔR_n (background-normalised fluorescence emission intensity) against cycle number is shown in Figure 3.12.

Table 3.11 Genes that are more than 3-fold upregulated in LPS treated THP1 cells compared to LPS + PC treated cells

Gene Name	GenBank ID	Function	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]	
			LPS + PC (Cy3)	LPS (Cy5)	LPS + PC (Cy3)	LPS (Cy5)	LPS/ LPS + PC	LPS + PC/ LPS
Macrophage inflammatory protein 1-beta (MIP-1β)	NM_002984	Chemokine (C-C)	16442	121751	34.51	234.50	6.79	0.15
Interleukin-8 (IL-8)	NM_000584	Chemokine (C-X-C)	9787	65497	20.54	126.15	6.14	0.16
Fibronectin leucine rich trans-membrane protein 3 (FLRT-3)	NM_013281	Cell adhesion/receptor signalling	376	2239	0.79	4.31	5.47	0.18
Macrophage inflammatory protein 4 (MIP-4)	NM_002988	Chemokine (C-C)	6922	41177	14.53	79.31	5.46	0.18
Macrophage inflammatory protein 3-alpha (MIP-3α)	NM_004591	Chemokine (C-C)	1209	7056	2.54	13.59	5.36	0.19
Interleukin-1 beta (IL-1β)	NM_000576	Inflammation	1823	10591	3.83	20.40	5.33	0.19
Glycoprotein hormones, alpha polypeptide (CG-α)	NM_000735	Common subunit of TSH, FSH, LH, CG glycoprotein hormones	398	2208	0.84	4.25	5.09	0.20
Macrophage inflammatory protein 1-alpha (MIP-1α)	NM_002983	Chemokine (C-C)	18768	103655	39.40	199.65	5.07	0.20
Connective tissue growth factor (CTGF)	NM_001901	Role in connective tissue formation and during wound healing	983	5169	2.06	9.96	4.83	0.21
BTG family, member 2 (BTG-2)	NM_006763	Cell cycle regulation	2535	13285	5.32	25.59	4.81	0.21
CD83 antigen (CD83)	NM_004233	Dendritic cell marker	2314	11100	4.86	21.38	4.40	0.23
Down syndrome critical region protein 1 (DSCR-1)	NM_004414	Calcineurin inhibition	2462	11641	5.17	22.42	4.34	0.23
CD38 antigen (p45) (CD38)	NM_001775	Metabolism of cADPR (Ca^{2+} messenger)	1077	4970	2.26	9.57	4.24	0.24

TSH = thyroid stimulating hormone; FSH = follicle stimulating hormone; LH = luteinising hormone; CG = chorionic gonadotrophin; cADPR = cyclic ADP-ribose.

[a] Calculated by dividing the normalised signal intensity for LPS treated cells by that for LPS + PC treated cells, and for LPS + PC treated cells by LPS treated cells.

Table 3.11 continued Genes that are more than 3-fold upregulated in LPS treated THP1 cells compared to LPS + PC treated cells

Gene Name	GenBank ID	Function	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]	
			LPS + PC (Cy3)	LPS (Cy5)	LPS + PC (Cy3)	LPS (Cy5)	LPS/ LPS + PC	LPS + PC/ LPS
Nuclear factor, interleukin 3 regulated (NFIL-3)	NM_005384	Regulation of IL-3 expression by T cells	1966	8737	4.13	16.83	4.08	0.25
Down syndrome critical region protein 1 (DSCR-1)	XM_048638	Calcineurin inhibition	2730	11960	5.73	23.04	4.02	0.25
GRO-1 oncogene (melanoma growth stimulating activity, alpha) (GRO-1)	NM_001511	Chemokine (C-X-C)	3986	17345	8.37	33.41	3.99	0.25
Serum/glucocorticoid regulated kinase (SGK)	NM_005627	Activation of certain potassium, sodium, and chloride channels	753	3252	1.58	6.26	3.96	0.25
Microrchidia (mouse) homologue (MORC)	NM_014429	Testis-specific, possible role in inhibiting apoptosis	474	2046	0.99	3.94	3.96	0.25
Coagulation factor III precursor (tissue factor, TF) (FIII)	NM_001993	Coagulation	668	2847	1.40	5.48	3.91	0.26
Tumor necrosis factor, alpha-induced protein 3 (TNF-αIP-3)	NM_006290	Termination of NFκB response	644	2693	1.35	5.19	3.84	0.26
DNAJ (HSP40) homologue, subfamily A, member 4 (DNAJA4)	NM_018602	Molecular chaperone homologue	2859	11717	6.00	22.57	3.76	0.27
Superoxide dismutase 2, mitochondrial (SOD-2)	NM_000636	Oxygen-free radical degradation	2583	10491	5.42	20.21	3.73	0.27
Regulated upon activation, normally T-expressed and presumably secreted (RANTES)	NM_002985	Chemokine (C-C)	2506	9995	5.26	19.25	3.66	0.27
Interleukin-23, alpha subunit p19 (IL-23α)	NM_016584	Stimulation of IFN-γ production	408	1593	0.86	3.07	3.58	0.28

[a] Calculated by dividing the normalised signal intensity for LPS treated cells by that for LPS + PC treated cells, and for LPS + PC treated cells by LPS treated cells.

Table 3.11 continued Genes that are more than 3-fold upregulated in LPS treated THP1 cells compared to LPS + PC treated cells

Gene Name	GenBank ID	Function	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]	
			LPS + PC (Cy3)	LPS (Cy5)	LPS + PC (Cy3)	LPS (Cy5)	LPS/ LPS + PC	LPS + PC/ LPS
Guanylate binding protein 2, interferon-inducible (GBP-2)	NM_004120	GTPase activity, signal transduction	618	2339	1.30	4.50	3.47	0.29
Tumor necrosis factor alpha (TNF-α)	NM_000594	Inflammation	6300	23371	13.22	45.01	3.40	0.29
Pleckstrin (PLEK)	NM_002664	Major PKC substrate of platelets	1066	3898	2.24	7.51	3.36	0.30
Spermidine/spermine N1-acetyltransferase (SAT)	NM_002970	Cell growth	2239	8170	4.70	15.74	3.35	0.30
Activating transcription factor 3 (ATF-3)	NM_004024	Transcription factor (repressor) increased in stimulated cells	453	1604	0.95	3.09	3.25	0.31
Receptor-interacting serine-threonine kinase 2 (RIPK-2)	NM_003821	Signal transducer for both innate and adaptive immune responses	1094	3785	2.30	7.29	3.17	0.31
Myeloid cell leukemia sequence 1 (BCL2-related) (MCL-1)	NM_021960	Anti-apoptotic	2041	7018	4.28	13.52	3.16	0.32
Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha (NFκBIA)	NM_020529	NFκB inhibition	12298	41275	25.82	79.50	3.08	0.32
Kinesin family member 4A (KIF-4A)	NM_012310	Transport	1518	4967	3.19	9.57	3.00	0.33

PKC = protein kinase C.

[a] Calculated by dividing the normalised signal intensity for LPS treated cells by that for LPS + PC treated cells, and for LPS + PC treated cells by LPS treated cells.

Table 3.12 Genes that are more than 2-fold downregulated in LPS-treated THP1 cells compared to LPS + PC treated cells

Gene Name	GenBank ID	Function	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]	
			LPS + PC (Cy3)	LPS (Cy5)	LPS + PC (Cy3)	LPS (Cy5)	LPS + PC/ LPS	LPS/ LPS + PC
PRO-0659 protein	NM_014138	Unknown	3687	339	7.74	0.65	11.84	0.08
Hypothetical protein	NM_017984	Unknown	1990	377	4.18	0.73	5.75	0.17
Neural cell adhesion molecule 1 (NCAM-1)	NM_000615	Cell-cell and cell-matrix adhesion	1535	427	3.22	0.82	3.92	0.26
Xeroderma pigmentosum, complementation group C (XPC)	NM_004628	DNA repair	1194	361	2.51	0.70	3.61	0.28
Toll-like receptor 9 (TLR-9)	NM_017442	Mediation of immune response to unmethylated CpG dinucleotides (i.e. bacterial DNA)	3512	1555	7.37	3.00	2.46	0.41
3'-phosphoadenosine 5'-phosphosulfate synthase 1 (PAPSS1)	NM_005443	Sulfotransferase enzyme co-substrate	729	347	1.53	0.67	2.29	0.44
Similar to phosphatidylinositol-4-phosphate 5-kinase, type I, beta (PIP5K1B)	XM_015261	Unknown	805	385	1.69	0.74	2.28	0.44
Mannan-binding lectin serine protease 2 (MASP-2)	NM_006610	Complement pathway activation	633	320	1.33	0.62	2.16	0.46

[a] Calculated by dividing the normalised signal intensity for LPS + PC treated cells by that for LPS cells, and for LPS cells by LPS + PC treated cells.

Table 3.13 Genes that are expressed in LPS + PC treated cells but show no expression in LPS-treated cells

Gene Name	GenBank ID	Function	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]
			LPS + PC (Cy3)	LPS (Cy5)	LPS + PC (Cy3)	LPS (Cy5)	
MAS-1 oncogene	NM_002377	Receptor, may modulate cell growth pathway	1324	123 [b]	2.78	0.24	4.63
Hypothetical protein	NM_025095	Unknown	791	203 [b]	1.66	0.39	2.77
cAMP-regulated guanine nucleotide exchange factor II (cAMP-GEFII)	NM_007023	Involvement in cAMP signalling independent of PKA, activation of Rap1 and 2	776	33 [b]	1.63	0.06	2.72
Deleted in lymphocytic leukemia, 1 (DLEU-1)	NM_005887	Putative tumour suppressor gene in B-cell chronic lymphocytic leukaemia	772	224 [b]	1.62	0.43	2.70
Fidgetin-like 1 (FIGNL1)	NM_022116	Possible ATPase	670	266 [b]	1.41	0.51	2.34
Abhydrolase domain containing 2 (ABHD2)	NM_007011	Membrane bound enzyme, function unknown	654	262 [b]	1.37	0.50	2.29
PRO-1085 protein, isoform 1	AL354776	Similar to protein serine/threonine phosphatase 4 regulatory subunit 1 (PPP4R1) (dephosphorylation of proteins)	648	232 [b]	1.36	0.45	2.27
Acyl CoA:monoacylglycerol acyltransferase 2 (MGAT2)	NM_025098	Possible role in dietary fat absorption	628	220 [b]	1.32	0.42	2.20
Defensin, alpha 3, preproprotein (DEFA3)	NM_005217	Located in microbicidal granules of neutrophils	605	96 [b]	1.27	0.18	2.12
Follicular lymphoma variant translocation 1 (FVT-1)	NM_002035	Located close to BCL-2 gene – possible involvement in tumoral process	595	248 [b]	1.25	0.48	2.08
Cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4) (CDKN2D)	NM_079421	Cell growth regulator	582	298 [b]	1.22	0.57	2.04

[a] Calculated by dividing the normalised signal intensity for LPS + PC-treated cells by 0.6. This was double the highest normalised intensity value displayed by an empty well and was taken as the cut-off point for no expression in the LPS-treated sample (0.6 corresponds to an intensity value of 312 bytes per pixel).

[b] Signal intensity below 'no expression' cut off point (Section 3.3.5), indicating no gene expression. This value can not therefore be used for calculation of gene expression ratio as results obtained would be unreliable due to slight fluctuations in background fluorescence between wells.

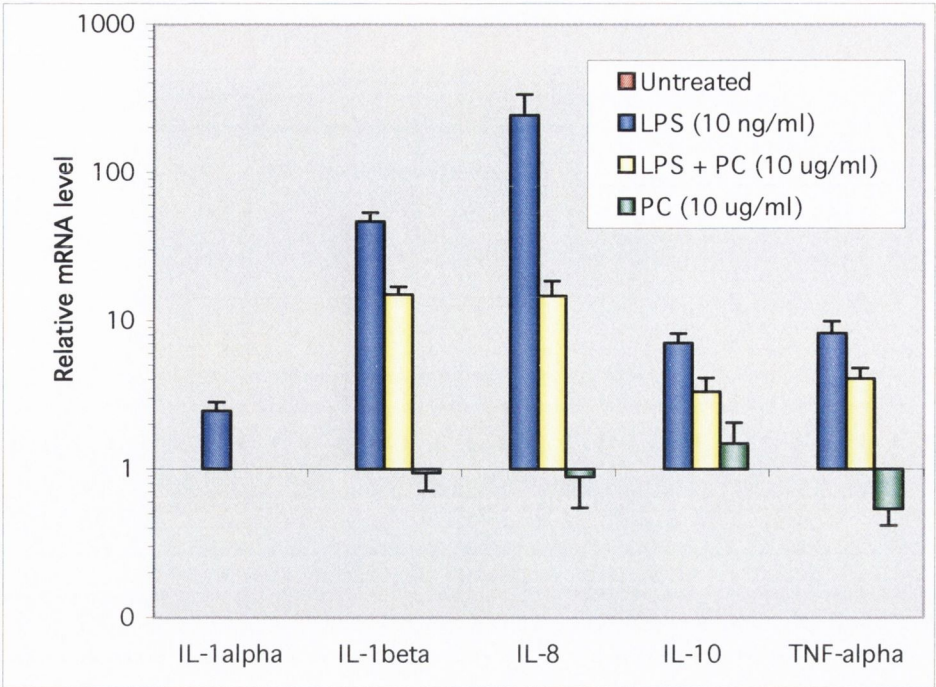

Figure 3.11 Relative cytokine gene expression in THP-1 cells following LPS and/or PC treatment. All expression levels are shown as fold difference relative to the untreated sample which was designated the calibrator and whose expression level was set at 1. However, for IL-1α, the untreated and PC treated samples showed no expression and thus the LPS+PC-treated sample was designated the calibrator. All values are averages of duplicate experiments, each analysed by real time PCR in duplicate, thus giving four sets of results. Error bars are the standard error of the four real time PCR results.

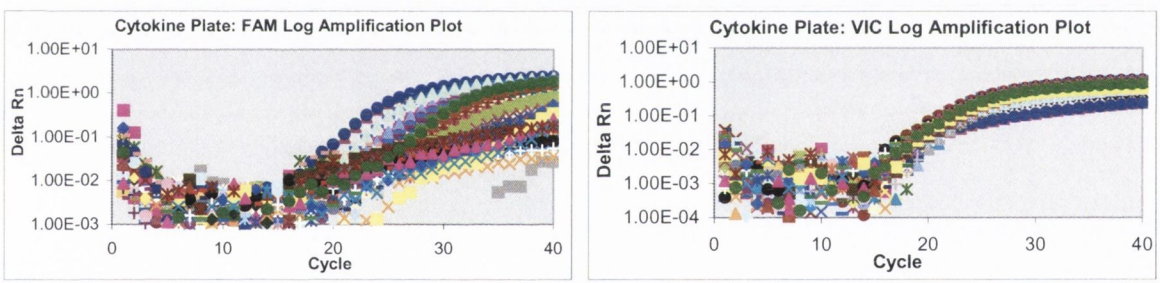

Figure 3.12 Logarithmic amplification plots of cytokine genes (FAM) and 18S rRNA gene (VIC) real-time PCR amplification. ΔR_n (background-normalised fluorescence emission intensity) is shown on the y-axis against cycle number on the x-axis.

The same cDNA was used to investigate IL-6 gene expression levels by real time PCR. These samples were designated R1, LPS1, R2, PC, L+PC, LPS2 (Section 3.2.1). No expression of the IL-6 gene was seen in the untreated samples (R1 and R2), the PC-treated sample (PC), or the LPS + PC-treated sample (LPS+PC). However, expression was seen in both LPS-treated samples (LPS1 and LPS2). Normalised Ct values (the IL-6 Ct value with the GAPDH endogenous control Ct value subtracted) of 13.78, 12.78, and 10.14 for LPS1, and 13.76, 12.44, and 12.34 for LPS2 were observed. Comparable results were seen with GAPDH as endogenous control. As no other samples expressed the IL-6 gene, relative gene expression calculation using a calibrator sample was not possible.

Relative levels of IL-8 and TNF-α mRNA were also determined using the GAPDH gene as the endogenous control (Figure 3.13). These results were comparable to those obtained with 18S rRNA as the endogenous control.

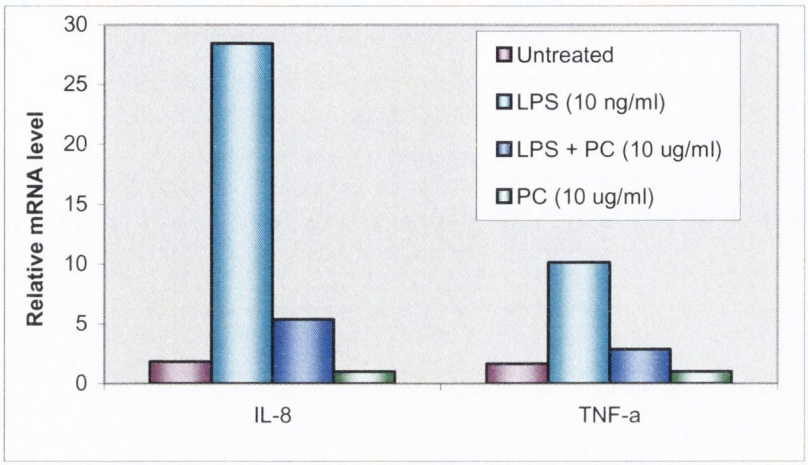

Figure 3.13 Relative IL-8 and TNF-α gene expression in THP-1 cells following LPS and/or PC treatment. Expression levels are shown as fold difference relative to the untreated sample which was designated the calibrator and whose expression level was set at 1. Results are average of triplicate real time PCR reactions.

Determination of Relative Cytokine Gene Expression in LPS, APC, and PC Treated Primary Monocytes by Real Time PCR

Isolated primary human monocytes were treated with 100 µg/ml APC or PC and 10 µg/ml LPS was added after 30 minutes. Control samples of untreated cells and LPS-treated cells were also included. Cells were incubated for 4 hours, after which time RNA was extracted and cDNA produced. The TaqMan Cytokine plate was used to determine relative gene expression of 12 cytokines in each sample. No expression of the IL-2, IL-4, IL-5, IL-12 (p35 and p40), IL-15, or IFN-γ genes was detected in any sample. Expression of IL-1 (α and β), IL-8, IL-10, and TNF-α was observed, and relative gene expression levels are shown in Figure 3.14.

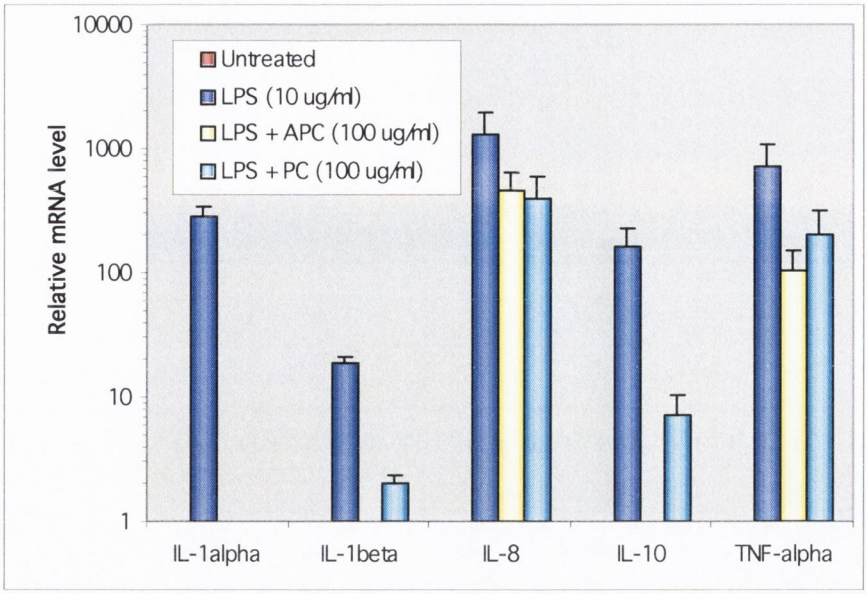

Figure 3.14 Relative cytokine gene expression in primary human monocytes following LPS and/or APC/PC treatment. For the IL-8, IL-10, and TNF-α genes, expression levels are shown as fold difference relative to the untreated sample which was designated the calibrator and whose expression level was set at 1. However, for IL-1α, the untreated sample and the L+APC-treated sample had no gene expression and thus the L+PC-treated sample was designated the calibrator. For the IL-1β sample, no gene expression was seen in the untreated sample and thus the L+APC sample was designated the calibrator. All values are averages of duplicate experiments, each analysed by real time PCR in duplicate, thus giving four sets of results. Error bars are the standard error of the four real time PCR results.

3.3.7 ELISA Analysis of Secreted Cytokine Protein Levels After LPS and APC/PC Treatment

In order to determine that the changes at the gene expression level of certain genes identified by microarray and real time PCR analysis were functional and lead to changes in protein levels, ELISA analysis was utilised to measure secreted levels of a range of cytokines in cell culture supernatants of THP-1 cells and of primary human monocytes treated under a variety of conditions as detailed below.

TNF-α Protein Levels

Secreted TNF-α levels from THP-1 cells and primary monocytes under varying treatment conditions were determined by ELISA. Results are shown in Figures 3.15 to 3.18.

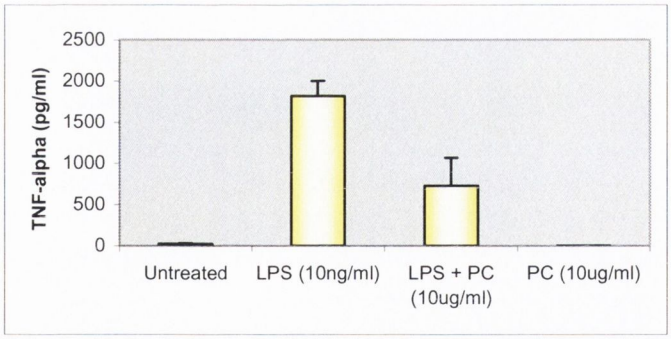

Figure 3.15 Effect of PC on TNF-α protein production by LPS-stimulated THP-1 cells. The supernatant used for TNF-α measurement was obtained from THP-1 cell cultures untreated or treated with LPS (10 ng/ml), LPS + PC (10 μg/ml), or PC (10 μg/ml). PC was added 30 minutes prior to LPS addition followed by 90 minutes incubation post LPS addition.

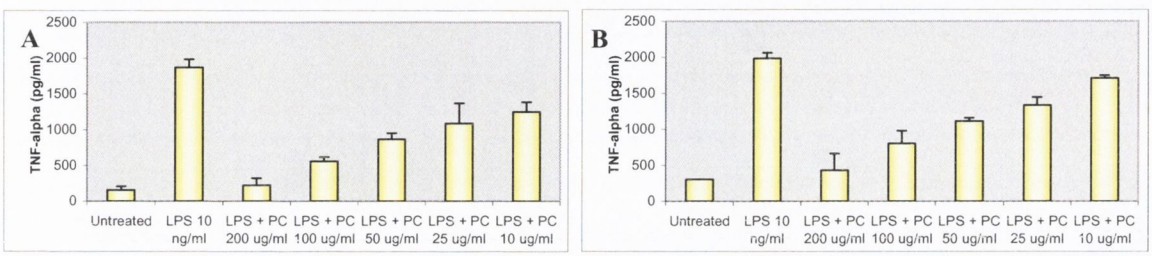

Figure 3.16 Effect of PC on TNF-α protein production by LPS-stimulated THP-1 cells. Cells were pre-incubated with different concentrations of PC for 30 minutes and then stimulated with LPS (10 ng/ml) for (A) 4 hours and (B) 24 hours. Supernatants were collected and TNF-α levels were determined by ELISA.

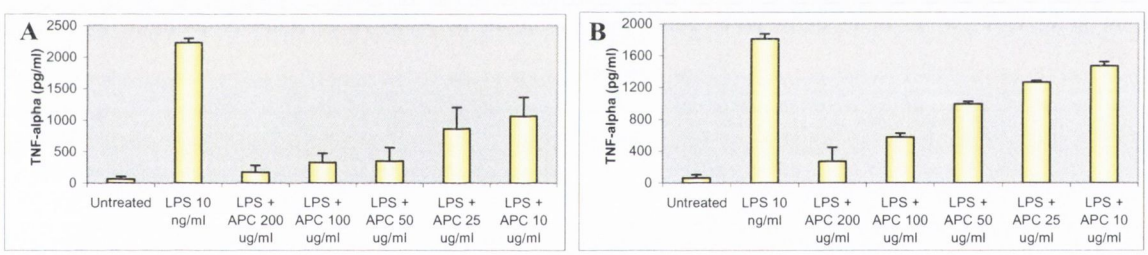

Figure 3.17 Effect of APC on TNF-α protein production by LPS-stimulated THP-1 cells. Cells were pre-incubated with different concentrations of APC for 30 minutes and then stimulated with LPS (10 ng/ml) for (A) 4 hours and (B) 24 hours. Supernatants were collected and TNF-α levels were determined by ELISA.

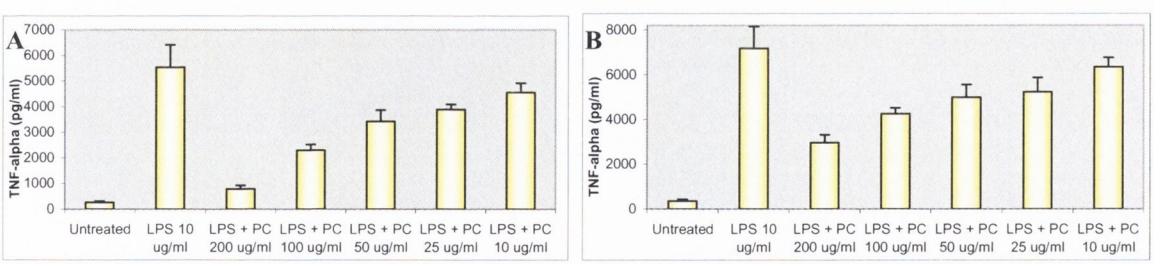

Figure 3.18 Effect of PC on TNF-α protein production by LPS-stimulated primary human monocytes. Cells were pre-incubated with different concentrations of PC for 30 minutes and then stimulated with LPS (10 μg/ml) for (A) 4 hours and (B) 24 hours. Supernatants were collected and TNF-α levels were determined by ELISA.

IL-6 Protein Levels

Secreted IL-6 levels from THP-1 cells and primary monocytes under varying treatment conditions were determined by ELISA. Results are shown in Figures 3.19 and 3.20. In contrast to 4 and 24 hour incubated samples, no IL-6 protein was detected in samples with a 90 minute incubation (after LPS addition), whether untreated, LPS treated, or LPS and/or PC treated.

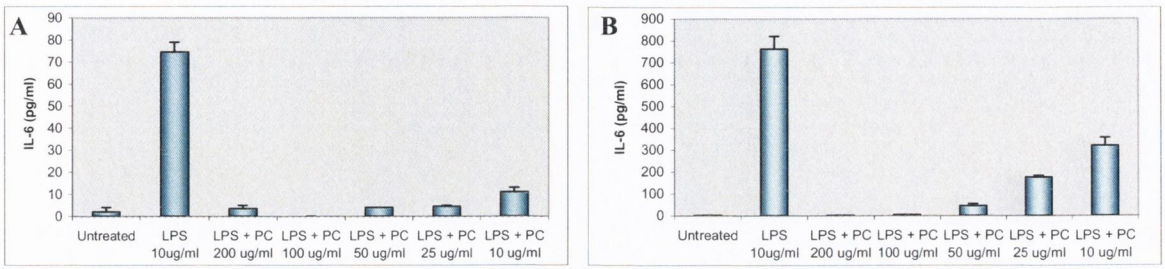

Figure 3.19 Effect of PC on IL-6 protein production by LPS-stimulated THP-1 cells. Cells were pre-incubated with different concentrations of PC for 30 minutes and then stimulated with LPS (10 μg/ml) for (A) 4 hours and (B) 24 hours. Supernatants were collected and IL-6 levels were determined by ELISA.

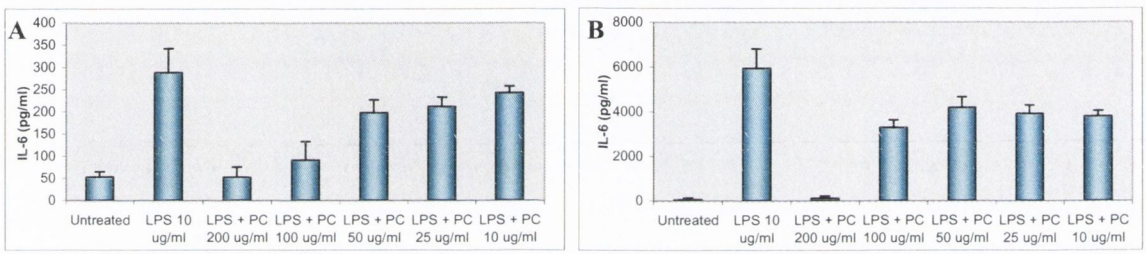

Figure 3.20 Effect of PC on IL-6 protein production by LPS-stimulated primary monocytes. Cells were pre-incubated with different concentrations of PC for 30 minutes and then stimulated with LPS (10 μg/ml) for (A) 4 hours and (B) 24 hours. Supernatants were collected and IL-6 levels were determined by ELISA.

IL-1β Protein Levels

Secreted IL-1β levels from THP-1 cells were determined by ELISA, as shown in Figure 3.21.

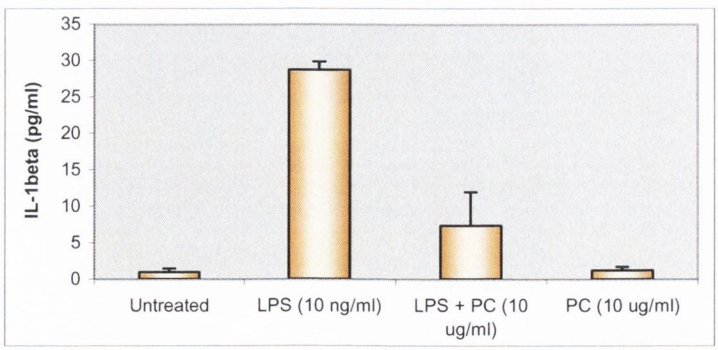

Figure 3.21 Effect of PC on IL-1β protein production by LPS-stimulated THP-1 cells. The supernatant used for IL-1β measurement was obtained from THP-1 cell cultures treated with LPS (10 ng/ml), LPS + PC (10 μg/ml), or PC (10 μg/ml). PC was added 30 minutes prior to LPS addition followed by 90 minutes incubation.

IL-8 Protein Levels

Secreted IL-8 levels from THP-1s were determined by ELISA, as shown in Figure 3.22.

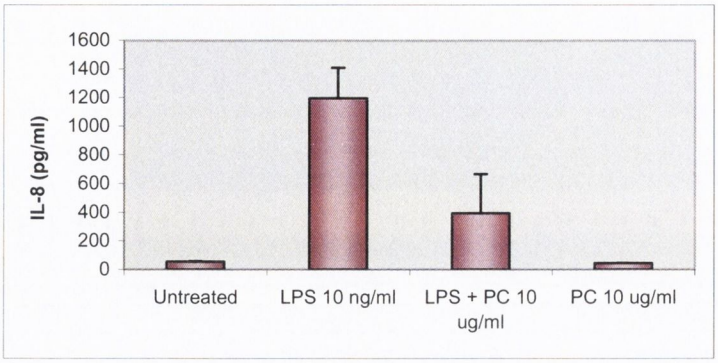

Figure 3.22 Effect of PC on IL-8 protein production by LPS-stimulated THP-1 cells. The supernatant used for IL-8 measurement was obtained from THP-1 cell cultures treated with LPS (10 ng/ml), LPS + PC (10 μg/ml), or PC (10 μg/ml). PC was added 30 minutes prior to LPS addition followed by 90 minutes incubation.

IL-10 Protein Levels

Secreted IL-10 levels from THP-1 cells and primary monocytes were determined by ELISA, as shown in Figures 3.23 and 3.24. In contrast to 24 hour incubated samples, no IL-10 protein was detected in samples with a 90 minute incubation (after LPS addition), whether untreated, LPS treated, or LPS and APC or PC treated.

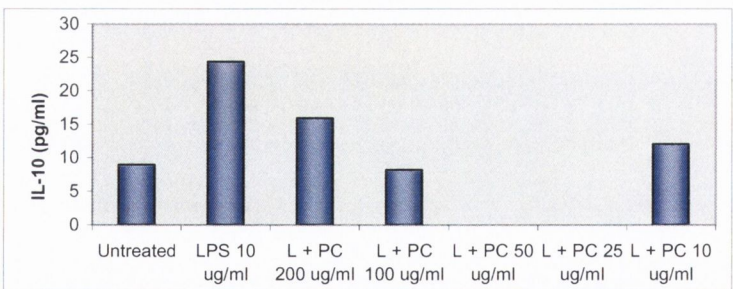

Figure 3.23 Effect of PC on IL-10 protein production by LPS-stimulated THP-1 cells. Cells were pre-incubated with different concentrations of PC for 30 minutes and then stimulated with LPS (10 μg/ml) for 24 hours. Supernatants were collected and IL-10 levels were determined by ELISA.

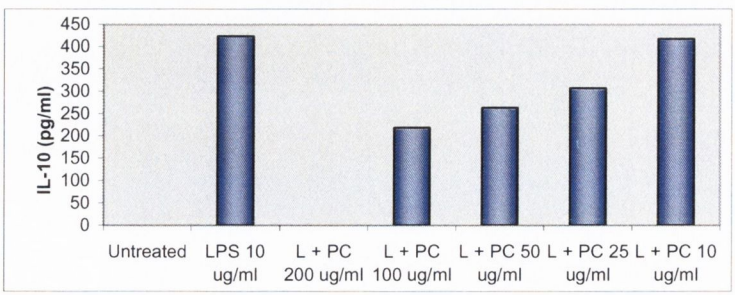

Figure 3.24 Effect of PC on IL-10 protein production by LPS-stimulated human primary monocytes. Cells were pre-incubated with different concentrations of PC for 30 minutes and then stimulated with LPS (10 μg/ml) for 24 hours. Supernatants were collected and IL-10 levels were determined by ELISA.

IL-4 and IFN-γ Protein Levels

No expression of IL-4 or IFN-γ was detected in either primary monocytes or THP-1 cells whether untreated, LPS-treated, and/or APC/PC-treated

Hirudin Treatment

The effect of hirudin, a potent thrombin inhibitor, on the downregulation of LPS-induced TNF-α secretion by APC and PC in THP-1 cells was investigated, as shown in Figure 3.25.

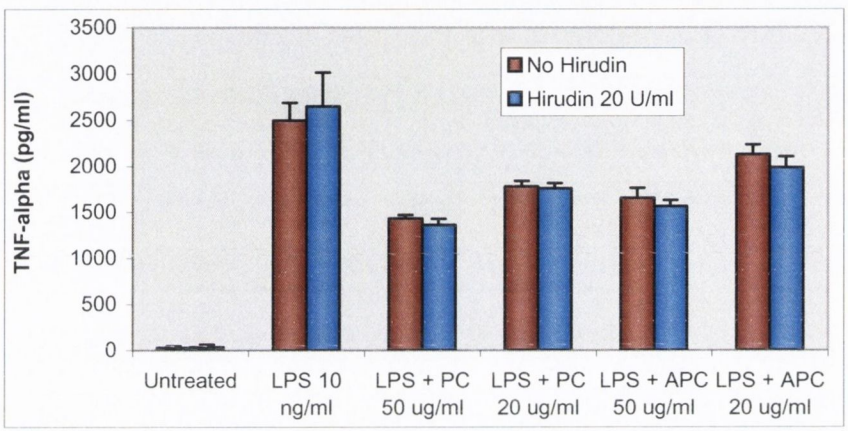

Figure 3.25 TNF-α protein production by THP-1 cells treated with hirudin (20 U/ml), APC or PC (50 μg/ml and 20 μg/ml), and LPS (10 ng/ml). APC or PC and hirudin were added 30 minutes prior to LPS addition followed by 4 hours incubation.

APC or PC Treatment Time Course Relative to LPS Addition

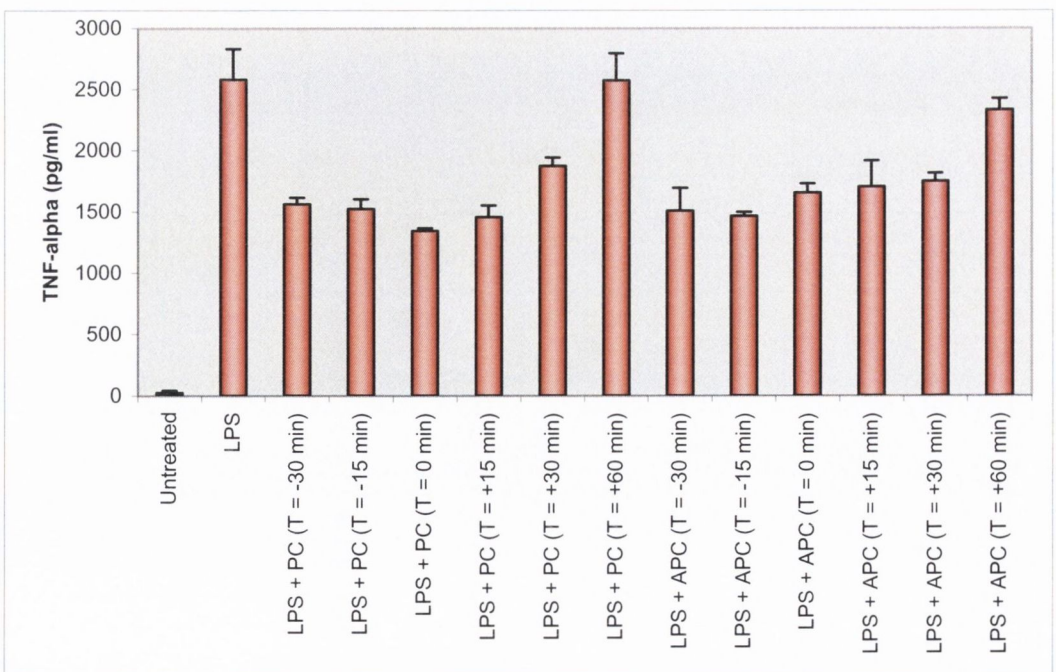

Figure 3.26 TNF-α levels in THP-1 cells ($1x10^6$/ml) untreated, LPS (10 ng/ml) treated, or LPS treated with APC or PC (50 μg/ml) added at varying time points relative to LPS addition. Total incubation time was 4 hours from LPS addition.

Samples treated with APC or PC alone

Treatment of either THP-1 cells or primary monocytes with either APC or PC (200 μg/ml to 10 μg/ml) alone had little effect on cytokine protein levels for any of the cytokines investigated (data not shown), i.e. equivalent results to untreated samples or lower/undetectable.

3.3.8 THP-1 Cell Viability and Proliferation after LPS and/or PC Treatment

In order to ascertain that any downregulation in gene expression observed when treating cells with PC was not due to cell death, THP-1 cell viability and proliferation under the experimental conditions used for microarray analysis was determined as outlined in Section 4.2.1. Results are shown in Figure 3.27.

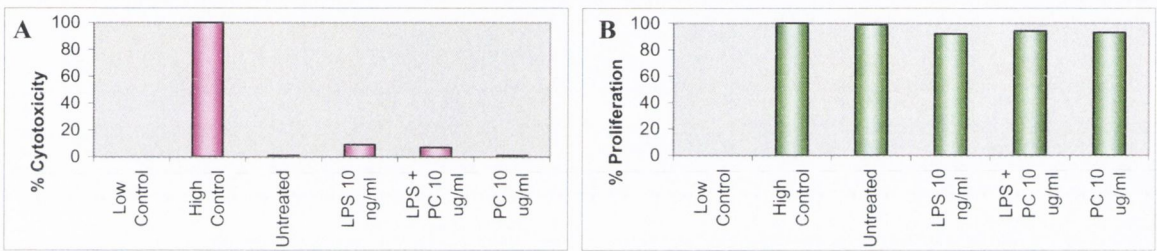

Figure 3.27 Quantitation of: (A) LPS and PC cytotoxicity on THP-1 cells by measurement of LDH release; and (B) THP-1 cell proliferation after LPS and PC treatment by measurement of WST-1 cleavage by mitochondrial dehydrogenases in viable cells. THP-1 cells (concentration 1 x 10^6/ml) were treated with the appropriate substance at the amount indicated per million cells for 2 hours (30 minute PC treatment followed by 1 ½ hour LPS treatment) before LDH release or WST-1 cleavage measurement. The low control for the cytotoxicity test was an untreated sample of THP-1 cells and a cell sample treated with 2% Triton x100 was used as the high control, whilst for the proliferation test the low control was a cell sample treated with 2% Triton x100 and the high control was an untreated sample of THP-1 cells.

3.4 DISCUSSION

During the development of endotoxin-induced sepsis, along with an upregulation of coagulation resulting in DIC, increasing amounts of pro-inflammatory cytokines are produced by stimulated monocytes leading to a range of symptoms including hypoperfusion, hypotension, and multiple organ failure. The association between acquired PC deficiency and sepsis (Fijnvandraat *et al*, 1995), the positive clinical response to APC replacement therapy that is observed in this disease (Bernard *et al*, 2001), and the cytokine driven multiorgan failure observed in septic shock all strongly suggest that PC may play a crucial role in the pathogenesis and treatment of severe sepsis. It is therefore highly important to understand the mechanism by which this protein exerts its effects. The role of the protein C pathway as an anticoagulant mechanism has been well documented over the past two decades. More recently, there is accumulating evidence of a critical role played by this pathway in the modulation of the inflammatory response to gram-negative bacterial infection. However, to date, our understanding of the mechanism of protein C involvement in the regulation of inflammation is limited, although recent work indicates that protein C binds to the monocyte cell surface and either directly or indirectly reduces transcription factor activity and thus downregulates the cytokine production that is associated with endotoxin stimulation of the monocyte.

Crucial to understanding the modulation of the inflammatory response by the protein C pathway is a detailed investigation of the genes that are upregulated and downregulated when LPS-stimulated monocytes are treated with APC or PC. Studies have shown on a small scale that LPS-induced cytokine production by monocytes is inhibited by APC or PC treatment (Grey *et al*, 1994; White *et al*, 2000b; Yuksel *et al*, 2002) and a larger study of the effect of recombinant APC on endothelial cells showed modulation of genes involved in the inflammatory and the cell survival pathways (Joyce *et al*, 2001). However, to date no large-scale gene expression study has been published that looks at the effect of PC treatment on the gene expression profile of LPS-stimulated monocytes. Given the central role of the monocyte in the pathogenesis of sepsis and associated disorders, the means by which the protein C pathway exerts its anti-inflammatory properties on this cell type is of importance. The aim of this study was to look at the gene expression profile of monocytes, both unstimulated and LPS-stimulated, and the effect of PC treatment on this profile. Thus RNA was extracted from the monocytic THP-1 cell line under varying treatment

conditions and the cDNA produced was fluorescently labelled and hybridised to microarrays representing approximately ten thousand human genes.

Cohen *et al* performed microarray analysis of the THP-1 response to *L. monocytogenes* infection (Cohen *et al*, 2000). They tested three commercially available high-throughput measurement systems (arrays) in parallel in order to determine the reproducibility and sensitivity of such systems. They observed close correlation between repeated measurements using either the same labelled sample and different arrays, or using independent samples prepared from the same cell pellet. They also determined that macroarray and Northern blot analysis showed a close correlation with DNA chip results. In large scale gene expression studies, changes in gene expression over 2-, 3-, and occasionally 4-fold are considered significant due to the assumption that low values are more difficult to reproduce. In our analysis, a fold difference of over 3-fold was considered significant, although genes with a 2-fold difference were also noted. Cohen *et al* used a fold change value of 1.8 as cut off for change in gene expression as they found this to be reproducible. They also found that intensity values less than 4 to 5 times the average noise signal were of poor reliability and should be discarded, and this correlates with the criterion used in our analysis of discarding intensity values 2-fold or less above the highest background level recorded, meaning that wells with intensity values below approximately 300 bytes per pixel were discarded as unreliable. This strict cut off point may cause signals and expression variations from genes expressed at low levels to be overlooked, but is, however, necessary in order to avoid false positive results. Finally, Cohen *et al* noted lower reproducibility for downregulated genes compared with upregulated genes. They found that 80% of down-regulated genes compared to 27% of up-regulated genes had low fold change (3 or less). They concluded that this could be due to the difficulty of reproducing low gene expression modulation. They also suggested that from a biological point of view, a decrease in mRNA is the result of complex events such as repression of basal transcriptional machinery, and mRNA turnover, and may be more difficult to highlight than an increase in mRNA levels due to induction of transcription, a positive regulatory event. We too detected a higher percentage of upregulated compared to downregulated genes especially in the unstimulated *versus* LPS-stimulated array. The LPS+PC- *versus* LPS-stimulated array was similar in that many genes were upregulated by LPS stimulation whereas the L+PC sample resembled the untreated sample in the first array, although in general these genes did not have as low an expression value as that seen in the untreated sample, presumably due to the influence of some LPS-induction which was dampened by

the presence of PC. In the PC-treated *versus* unstimulated array, very few genes were either up- or downregulated by PC-treatment indicating little effect of the zymogen on monocytes in the resting state.

Initially, cDNA obtained from unstimulated cells was hybridised to a microarray slide in conjunction with that from LPS-stimulated cells, in order to determine the level of gene upregulation or downregulation and the categories of genes affected by endotoxin stimulation of the THP-1 cell. No study has investigated the gene expression profile of LPS stimulated THP-1 cells. Studies that have been undertaken include a gene profile of LPS stimulated primary human monocytes by serial analysis of gene expression (SAGE) (Suzuki *et al*, 2000), a small scale (96 gene) analysis of LPS stimulated MM6 cells (a monocyte cell line) (Heller *et al*, 1997), microarray analysis of the THP-1 response to *L. monocytogenes* infection (Cohen *et al*, 2000), microarray analysis of LPS-treated neutrophils (Malcolm *et al*, 2003), microarray analysis of LPS-treated HUVECs (human endothelial cell line) (Zhao *et al*, 2001), and microarray analysis of PHA-responsive genes in peripheral blood mononuclear cells (PBMCs) (Walker and Rigley, 2000).

As expected, the expression of a large number of genes was upregulated by LPS-stimulation as a result of the LPS binding to the cell and signalling to activate a range of transcription factors. Of the genes upregulated 3-fold or more by LPS, one of the larger groups consisted of those encoding chemokines. The expression of IL-8, a chemokine and one of the major mediators of the inflammatory response, was increased 44 fold compared to levels in the resting cell, and this upregulation was confirmed by real-time PCR and at the functional level by ELISA analysis. This increase was accompanied by an upregulation of other chemokines such as MIP-4, MIP-1β, MIP-3α, and MIP-1α, which all increased by more than 10-fold, GRO-1 and RANTES were both increased over 3-fold. This stimulated increase in chemokine expression has been demonstrated in a range of studies including SAGE analysis of the effect of LPS stimulation on primary human monocytes (Suzuki *et al*, 2000), the *in vitro* (macrophages) and *in vivo* (mouse) LPS stimulation of chemokine mRNA and protein levels (Kopydlowski *et al*, 1999), and the other gene profile studies mentioned above. Thus upregulation of chemokine gene expression appears to be a general response to a variety of inflammatory stimuli in several cell types.

The primary pro-inflammatory cytokines IL-1β and TNF-α were also increased by LPS stimulation of THP-1 cells, as was IL-23α which stimulates IFN-γ production. Cytokines

such as IL-1α and IL-1β, IL-6, GM-CSF, and TNF-α are increased in LPS-stimulated MM6 cells (Heller *et al*, 1997); IL-1β, IL-6, and TNF-α are increased in LPS stimulated primary human monocytes (Suzuki *et al*, 2000); and IL-1β and TNF-α are increased in *L. monocytogenes* infected THP-1 cells (Cohen *et al*, 2000). Whilst we saw a significant increase in IL-1β and TNF-α levels, it was of interest to note that IL-6 expression was not markedly changed, and there was in fact no observed expression of IL-6 in either resting or LPS-stimulated THP-1 cells. IL-6 was one of the most highly upregulated genes in the primary monocyte SAGE study (Suzuki *et al*, 2000). However, we found that IL-6 is not upregulated by LPS stimulation to a great extent in THP-1 cells compared to primary human monocytes, as shown in our real time PCR and protein analysis of IL-6 levels, and therefore the microarray analysis may not have been sensitive enough to detect any increase in expression of this gene. The gene profile analysis of THP-1 cells infected with *L. monocytogenes* did not count IL-6 amongst genes upregulated by the infection despite a large increase in both IL-1β and TNF-α gene expression (Cohen *et al*, 2000). This serves to highlight the potential variations between different cell lines and between cell lines and primary human cells, as discussed later in this section.

NF-κB, the pivotal transcription factor in the activation of LPS-induced genes, was also increased upon LPS stimulation. However, it must be noted that IκBα (or NF-κBIA), inhibitor of NF-κB, was also upregulated by LPS. NF-κBIA and NF-κBIB inhibit NF-κB by binding to it and trapping it in the cytoplasm. However, IKKα and IKKβ, IκB kinases, are intracellular targets for LPS (Hawiger *et al*, 1999). LPS therefore does not exert its NF-κB activation through downregulation of IκB gene expression, rather through degradation of IκB by IKKα and IKKβ, allowing NF-κB translocation to the nucleus. This increase in IκBα seen in monocytes upon LPS stimulation may be an attempt by the cell to regulate the inflammatory response driven by the increased NF-κB translocation to the cell nucleus. In microarray analysis of an LPS stimulated monocytic cell line, MM6, IκB expression was also stimulated by LPS, and was found to be increased to a greater degree than NF-κB gene induction, as was found in our study (Heller *et al*, 1997). Other genes involved in signal transduction were also induced by LPS. Growth arrest and DNA-damage-inducible-α (GADD-45α) which activates the p38/JNK signal transduction pathway, and Fos, which dimerises with the Jun family to form the transcription factor AP-1, were both induced. Receptor interacting serine-threonine kinase 2 (RIPK-2/RIP-2), a transducer of signals from receptors of both the innate and adaptive immune responses (Kobayashi *et al*, 2002), was upregulated, and the NFE-2L2 transcription factor was also induced.

The coagulation factor III gene, which encodes tissue factor (TF), was induced by LPS stimulation. As discussed in chapter 1, TF is a major initiator of the coagulation pathway, leading to production of thrombin and the formation of a fibrin clot. The induction of this gene by LPS further demonstrates the link between the coagulation and inflammation responses. Connective tissue growth factor (CTGF) which plays a role in fibrotic connective tissue formation and during wound healing was also induced by LPS (Workalemahu *et al*, 2003), as was the cytoskeletal element vimentin (VIM), an intermediate filament protein involved mainly in structural processes such as wound healing. VIM is secreted by activated macrophages and is involved in bacterial killing and generation of oxidative metabolites in addition to its role as a cytoskeletal element (Mor-Vaknin *et al*, 2003).

Upon LPS monocyte stimulation, several anti-inflammatory genes were induced as well as the pro-inflammatory cytokine and chemokine gene induction discussed above. Examples of such anti-inflammatory genes induced by monocyte LPS stimulation are TNF-α-induced protein 6 (TNF-αIP6/TSG-6), which acts as an anti-inflammatory protein by activating inter-α-inhibitor which has anti-plasmin and anti-inflammatory properties (Fries and Kaczmarczyk, 2003), DSCR-1 as discussed in the next paragraph, and annexin A, which inhibits phospholipase A2, a protein required for the biosynthesis of prostaglandins and leukotrienes, both potent mediators of inflammation. Superoxide dismutase 2 (SOD-2) is the mitochondrial isoform of SOD whose function is the degradation of superoxide, a reactive oxygen species (ROS). ROS are the toxic by-products of oxidative phosphorylation which have been implicated in a wide variety of degenerative processes. Superoxide promotes vasoconstriction, damage to the endothelium, adhesion molecule expression, and thus perpetuation of inflammation. LPS, IL-1α, and TNF-α promote superoxide formation in cultured vascular tissues through an upregulation of NADPH oxidase activity (Muzaffar *et al*, 2003). Thus the increase of SOD-2 expression in response to LPS is likely to act as a regulator of the effects of superoxide. The induction of these anti-inflammatory genes points to auto-regulation of the monocyte inflammatory response, although a clearer picture of the amount of induction of such genes and the stage of induction in the inflammatory response could be obtained by looking at the course of induction of pro- and anti-inflammatory gene expression over time. For example, Walker *et al* looked at PHA responsive genes at a range of time points (1hr, 6hr, 12hr, and 24hr) and noted five gene clusters based on their temporal expression profiles (Walker and

Rigley, 2000). Certainly, we did not see expression of IL-10 either at the mRNA or protein level in cells incubated with LPS for 90 minutes, although IL-10 protein expression, determined by ELISA, was seen after 24 hours incubation with LPS. IκB, the NF-κB inhibitor, whose gene expression was also increased, can further be included in the genes that possibly play a regulatory role in the cellular response to LPS.

As well as some of the well-known LPS-induced genes, our microarray analysis demonstrated the involvement of a number of genes whose roles are poorly or not understood in monocyte activity during the inflammatory response. The Down syndrome critical region protein-1 (DSCR-1) gene was upregulated by LPS. However, this gene was represented by two spots on the array, one of which showed a 26 fold increase in LPS stimulated cells, whilst the second showed a 3.17 fold increase compared to resting cells. This difference was mainly due to a large difference in the unstimulated sample (a signal intensity of 469 bytes per pixel for the XM_048638 spot and 3188 for the NM_004414 spot), whereas LPS-stimulation resulted in more uniform expression (signal intensities of 10656 and 8534 respectively). These somewhat different results allow the conclusion that expression of DSCR-1 is increased by LPS stimulation, yet they also highlight the need to verify any results from such an analysis. The DSCR-1 gene was also identified as a PHA-responsive gene in PBMCs (Walker and Rigley, 2000), although it was found to fall to below resting levels 12 hours after PHA stimulation after an increase in levels over the initial six hours. This protein binds and inhibits calcineurin (also known as protein phosphatase 2B or PP2B), a serine-threonine phosphatase which mediates many cellular responses that use calcium signalling, including activation of T and B lymphocytes, where activation through antigen receptors induces calcineurin dependent targeting of nuclear factor of activated T cells (NFAT) proteins to their binding sites in the TNF-α gene promoter. However, calcineurin phosphatase activity is not required for LPS-stimulated TNF-α gene expression in macrophages (Tsai *et al*, 2000). The NFAT sites in the TNF-α gene promoter that are required for induction of the gene by calcineurin-dependent stimuli in lymphocytes are functional binding sites for the ERK-targeted Ets and Elk proteins in LPS-stimulated TNF-α induction in macrophages, meaning that in LPS-stimulated macrophages TNF-α gene expression is unaffected by the upregulation of DSCR-1. The function of LPS-induced DSCR-1 upregulation in monocytes by LPS stimulation is unclear. It would act to suppress activation in T cells and thus may play a role in restraining the inflammatory response, however this role appears to be redundant in monocytes where TNF-α activation can take place by an alternative route.

Spermidine/spermine N1-acetyltransferase (SAT), whose function is to regulate the polyamine metabolic pathway, was also induced by LPS stimulation. Polyamines are thought to play several important roles in cellular regulation, growth, and differentiation. SAT regulates polyamine metabolism by the N1-acetylation of spermidine or spermine which is the rate-limiting enzymatic step of this pathway (Casero and Pegg, 1993). Spermine inhibits pro-inflammatory cytokine synthesis in human mononuclear cells (Zhang *et al*,), a possible counter-regulatory mechanism to restrain the innate immune response, and thus increased SAT is likely to reduce the amount of spermine and therefore promote monocytic cellular activation, although the decrease in intracellular polyamines associated with decreased spermine may also reduce cellular growth rates. Increased SAT expression was also seen in LPS treatment of endothelial cells (Zhao *et al*, 2001).

Other genes induced by LPS included the extracellular matrix protein FLRT-3. This protein is thought to be involved in cell adhesion and receptor signalling and thus may play a role in the attachment and mobilisation of other immune effector cells. The CG-α gene encodes the alpha subunit of the glycoprotein hormones. This subunit is identical in chorionic gonadotrophin (CG), luteinising hormone (LH), follicle stimulating hormone (FSH), and thyroid stimulating hormone (FSH), whereas the β subunit is unique in each of these hormones and confers biological specificity. These hormones regulate major metabolic and reproductive functions of the body. The possible function of this increase of CG-α expression in response to LPS is not clear. Serum- and glucocorticoid-regulated kinase (SGK), is involved in activating certain potassium, sodium, and chloride channels, and is thought to play a role in blood pressure regulation, especially hypertension (Busjahn *et al*, 2002). A homologue of DNAJ (heat shock protein 40/HSP40), a molecular chaperone, was also induced by LPS. Molecular chaperones recognise and renaturate misfolded proteins under normal and stressed conditions. CD38, originally used as a marker for lymphocyte differentiation but now known to be expressed in many cells, is a multifunctional enzyme responsible for metabolising cyclic ADP-ribose (cADPR), a mediator of Ca^{2+} signalling. Its physiological functions are widespread and include cell proliferation and involvement in neutrophil chemotaxis (Musni *et al*, 2002). CD83 is a marker for mature dendritic cells (DCs), and inhibits DC-mediated T-cell proliferation (Lechmann *et al*, 2002). The induction of expression of this marker on monocytes possibly indicates differentiation of some monocytes into mature DCs (Zhou and Tedder, 1996). KIF4A is a member of the kinesin superfamily of transporter molecules. The transport

target for human KIF4A is unknown, although the mouse homologue is a transporter of membranous organelles (Oh *et al*, 2000). Dual specificity phosphatase 1 (DUSP-1) is thought to play a role in the cellular response to environmental stress and negatively regulate cellular proliferation (Keyse and Emslie, 1992). Zinc finger protein 133 is a transcriptional regulatory protein containing two zinc finger domains. The functional role is unclear but this class of proteins may be involved in cellular proliferation and differentiation. B-cell translocation gene 2 (BTG2) belongs to a family of genes with anti-proliferative properties and BTG2 is involved in regulation of the G1/S phase of the cell cycle (Rouault *et al*, 1996). Ribosomal proteins S13 and L37 are components of the 40S and 60S ribosomal subunits respectively, and are thus components of the translational machinery which catalyses new protein synthesis. Regulator of G protein signalling 2 (RGS-2) mRNA is consistently observed in acute leukaemia patient samples indicating a role in leukogenesis or a common consequence of dysregulated cell growth (Wu *et al*, 1995). RGS-2 expression is markedly increased in human B cells exposed to various mitogens and cytokines (Siderovski *et al*, 1994). The cholinergic receptor, nicotinic, alpha polypeptide 7 (CHRNA-7) forms a nicotinic receptor which opens an ion-conducting channel across the plasma membrane upon acetylcholine binding. The α7 nicotinic acetylcholine receptor is expressed in leukocytes but it does not bind nicotine or acetylcholine and thus its physiological role in leukocytes remains unknown (Villiger *et al*, 2002).

Two genes that are possibly involved in regulation of apoptosis, both anti-apoptotic, were upregulated in LPS-stimulated cells. Nuclear factor IL-3 regulated (NFIL-3) controls a pivotal step in an anti-apoptotic signalling pathway in B lymphocytes (Ikushima *et al*, 1997) and regulates IL-3 expression by T cells. The human homologue of mouse MORC protein is reported to be testis-specific, thus it is unclear why transcription of this gene is increased in LPS-stimulated monocytes. MORC knockout mice show greatly increased numbers of apoptotic cells in the testis, indicating that this protein may play a role in regulating apoptosis (Watson *et al*, 1998).

Our results demonstrate that monocytes respond to LPS by regulating their gene expression in such a way that a network of pathways is activated, co-operating to counteract the presence of LPS in the bloodstream. Genes involved in inflammation, coagulation, cellular differentiation, cellular proliferation, cellular response to environmental stress, transcriptional activation, signal transduction, and cell survival are amongst the genes

induced by LPS stimulation of monocytes. The genes upregulated by LPS treatment of THP-1 cells mirror some of the genes altered in other gene expression profile studies investigating either different cell types, different stimulants, or both (Suzuki *et al*, 2000; Heller *et al*, 1997; Cohen *et al*, 2000; Malcolm *et al*, 2003; Zhao *et al*, 2001). However many differences in expressed genes were also seen between these studies suggesting that cells of different tissues respond differently to whole bacteria or bacterial cell wall products or that different bacteria or their products may differentially affect expression of mammalian cells (Zhao *et al*, 2001).

Only a few genes were downregulated more than 3-fold by LPS treatment. The thioredoxin interacting protein (TXNIP) is an inhibitor of thioredoxin, a major regulator of cellular redox state. TXNIP is thought to be an anti-tumour gene which forms a transcriptional repressor complex (Han *et al*, 2003). Neural cell adhesion molecule 1 (NCAM-1) (also known as CD56), a member of the immunoglobulin superfamily, is a membrane bound glycoprotein that plays a role in cell-cell and cell-matrix adhesion. It regulates interactions between neurons and between neurons and muscle. It is expressed by natural killer and neuroendocrine cells, and is also expressed by cells of various human malignancies (Zeromski *et al*, 2001) and is thus thought to be involved in tumour biology, however it is not known to be expressed by monocytes. The MAS-1 oncogene encodes a receptor that when activated may regulate a critical component in the growth-regulating pathway to bring about oncogenic effects (Young *et al*, 1986). Three genes of unknown function were also downregulated by LPS. The association of the three genes with known function to tumour biology indicates that these results might be linked to the cancerous nature of cell lines rather than to the immune response of these cells to LPS. It is also of interest to note that NCAM-1, MAS-1, and two of the three proteins of unknown function are downregulated in LPS treated cells compared to LPS + PC treated cells, which is further proof of the similarity of the LPS + PC treated cell gene profile to that of resting cells. However, these four genes are also downregulated in PC treated THP-1 cells compared to untreated cells, indicating that the reduction in expression of these genes may be a general response to the addition of an extraneous compound such as LPS or PC to the cell culture.

Following the microarray comparison of untreated THP-1 cells with LPS stimulated cells, the gene expression profile of LPS stimulated cells was compared with that of cells treated with PC and LPS. The general pattern of gene expression observed under these conditions was a downregulation of all LPS-induced genes in the cells treated with PC prior to LPS

addition. Twenty-eight genes that were upregulated more than 3 fold in the LPS *versus* untreated array were downregulated more than 3 fold by PC pre-treatment in the LPS+PC *versus* LPS array. Of the other fifteen genes that had been upregulated more than 3 fold by LPS, all were downregulated more than 2 fold by PC pre-treatment apart from one gene of unknown function (NM_024972) and the two ribosomal proteins (S13 and L37) which were downregulated 1.89, 1.65, and 1.63 fold respectively in LPS+PC treated compared to LPS treated cells. Conversely, five genes were included in the set of genes downregulated more then 3 fold in LPS+PC compared to LPS-treated cells that had not been upregulated more than 3 fold by LPS compared to untreated cells, implying that they were decreased by PC more than they were upregulated by LPS. These included three genes that had been upregulated more than 2 fold by LPS. These were TNFαIP-3 and guanylate binding protein 2 (GBP-2) upregulated 2.95 and 2.8 fold by LPS treatment, and myeloid cell leukaemia 1 (MCL-1) upregulated just 2.11 fold by LPS treatment compared to unstimulated cells. The other two genes encoded plekstrin, a major substrate of protein kinase C in platelets, which was upregulated 1.75 fold by LPS, whilst gene expression of activating transcription factor 3 (ATF-3), a transcription factor, was increased in the LPS sample of the LPS+PC *versus* PC array, however expression of both samples in the untreated *versus* LPS array was very low, indicating that LPS did not up-regulate ATF-3 gene expression as it did in the LPS sample used for the LPS+PC *versus* LPS array.

The process of inhibition of LPS-stimulated gene expression in cells treated with PC is still to be fully elucidated. Several possible mechanisms of PC action are possible. PC may block LPS binding to its receptors or it may block the signal transduction pathways induced by this binding. PC may also act by down-regulating the cell surface expression of the LPS receptors such as TLR4 and CD14. PC is known to inhibit NF-κB translocation to the cell nucleus (White *et al*, 2000b) and in fact Zhao *et al* demonstrated that in LPS-treated HUVEC cells, all LPS-stimulated genes were no longer upregulated when an inhibitor of NF-κB translocation was used (Zhao *et al*, 2001). Translocation of EPCR (with APC bound) from the plasma membrane to the nucleus where it may re-direct gene expression has also been put forward as the mechanism by which APC may modulate inflammatory mediator responses (Esmon, 2000a). It is clear that PC does not work by inducing anti-inflammatory gene expression, since several anti-inflammatory genes whose expression is induced by LPS stimulation are also decreased by PC treatment prior to LPS addition. Certainly IL-10, which inhibits IL-1β, IL-6, IL-8, and TNF-α production in monocytes by blocking NF-κB, has auto-regulatory properties (Wang *et al*, 1995; de Waal

Malefyt *et al*, 1991). From this evidence, it appears that IL-10 expression is regulated in the same way as the expression of TNF-α, IL-6, and IL-1β and thus PC would have the same effect on its expression.

A variety of chemokines were amongst the genes most highly upregulated by LPS stimulation of monocytes, and these were all downregulated by PC treatment. Furthermore, recent publications have demonstrated the effectiveness of PC and APC in inhibiting monocyte, neutrophil, and eosinophil chemotaxis (Sturn *et al*, 2003; Feistritzer *et al*, 2003). Therefore, chemotaxis and chemokines appear to be of major importance in the cell response to inflammatory stimuli and also in the mechanism of anti-inflammatory activity of APC. Chemotaxis is of major importance in the innate immune response since the recruitment of inflammatory mediator cells, especially monocytes/macrophages and neutrophils, to the site of injury is crucial in the host response to tissue injury or bacterial invasion. However, excessive chemotaxis of immune cells is of clinical importance in severe sepsis and related conditions such as ARDS where leukocytes accumulate in the lungs. In baboons exposed to *E. coli*, there is an influx of leukocytes into the adrenal, renal, and hepatic microvasculature (Taylor *et al*, 2000) and in LPS-induced pulmonary vascular injury in rats leukocytes are quickly recruited to the lungs (Murakami *et al*, 1996). Thus a major component of the protective effect mediated by APC in sepsis is the reduction of chemokine synthesis and the inhibition of chemotaxis and recruitment of the principle inflammatory mediator cells, neutrophils and monocytes, to sites of injury or inflammation.

The third gene expression profile comparison was between unstimulated and PC treated cells. This resulted in very few changes in gene expression. Only two genes were upregulated more than 3 fold in PC-treated cells compared to untreated cells. INE-1, highly upregulated in PC-treated cells, is expressed from both X chromosomes in the female rather than undergoing inactivation where only one copy of the gene is transcribed. The function of this gene is unknown. Further investigation as to the effect of PC on its expression is needed in order to determine if this is consistently a PC stimulated gene and what function it serves. The second upregulated gene, located on chromosome 11, encodes a nuclear protein of unknown function. Genes downregulated by PC included four genes also downregulated by LPS: two proteins of unknown function, NCAM-1, and MAS-1. These were discussed earlier. Endomucin-2 expression was downregulated by PC treatment, with high expression in the untreated sample. However, expression of this gene in all other array samples (untreated, LPS, LPS+PC, and LPS2) was undetectable,

suggesting that the high expression of this gene in this untreated sample might be a spurious result and needs to be further investigated to establish the effect of PC on its expression. Very little information was gained from the results of this microarray slide, and the general conclusion is that PC treatment alone has little effect on the gene expression profile of monocytes. Since the dose of PC administered was close to the normal plasma levels of PC this conclusion is perhaps unsurprising and highlights the specific role for PC in modulating the activated monocyte.

It should be noted that Cohen *et al* reported much higher fold upregulation of a number of genes, including pro-inflammatory cytokines, than was seen in our analysis of LPS-stimulation of THP-1 cells (Cohen *et al*, 2000). This may reflect a difference between the effect of LPS and *L. monocytogenes* infection on THP-1 cells. On the other hand it may indicate that our gene profile analysis was less sensitive in detecting changes in gene expression and we may therefore be detecting the highly induced genes, but ones that are not increased to a large extent may be masked by the background noise in the array. This could especially be the case in the array comparing unstimulated to PC-treated cells in which our analysis may not have been sensitive enough to detect any influence of PC treatment on gene expression. Thus although the ability of arrays may not be as great as that of RT-PCR to detect fold changes in gene expression, their ability to globally identify candidate genes for involvement in cellular perturbations nonetheless makes them an important tool in understanding processes involving transcription activation (Zhao *et al*, 2001).

Despite the previous finding that PC protects endothelial cells from apoptosis (Joyce *et al*, 2001; Mosnier and Griffin, 2003), we did not see an increase in anti-apoptotic gene expression or decrease in pro-apoptotic gene expression in monocytes following PC treatment. However, both APC and PC have no effect on apoptosis in eosinophils (Feistritzer *et al*, 2003) or neutrophils (Sturn *et al*, 2003). Sturn *et al* noted that the persistence of a neutrophil-mediated inflammatory response is due in part to a delay in their spontaneous rates of apoptosis or cell death and thus the stimulation of apoptosis would have important implications for the resolution of inflammatory disorders. Therefore the observation that inhibition of apoptosis with APC or PC as reported in endothelial cells was not observed with neutrophils is compatible with the anti-inflammatory action of PC. A similar role for apoptosis in attenuating the inflammatory response mediated by monocytes/macrophages could be important, and thus the finding that PC had no effect on

apoptosis related genes ties in with its anti-inflammatory effects on monocytes. In fact, the two genes upregulated by LPS, NFIL-3 and MORC, that were associated with apoptosis are both thought to be anti-apoptotic, and expression of both of these was downregulated by PC treatment of LPS stimulated cells. Additionally, the anti-apoptotic gene MCL-1, was induced just 2.1 fold by LPS stimulation compared to untreated cells, but gene expression was decreased over 3 fold in LPS+PC-treated cells compared to LPS-treated cells. The monocyte apoptotic gene findings shown here, along with those from studies looking at the effect of PC on apoptosis and chemotaxis in neutrophils and eosinophils (Feistritzer *et al*, 2003; Sturn *et al*, 2003), highlight the biological difference between these leukocytes and endothelial cells in relation to the effect of the protein C pathway. This is of interest as it suggests that a housekeeping system such as the protein C pathway may have different housekeeping activities in different cell systems, in this case particularly in relation to apoptosis and chemotaxis.

There has been much debate as to the possible superiority of APC over its zymogen PC in the treatment of severe sepsis (Faust *et al*, 2001; de Kleijn *et al*, 2003). PC was utilised in our investigation as it was more readily available and available in greater amounts than APC, and a considerable quantity of PC was needed in order to treat the high number of THP-1 cells necessary to obtain enough RNA for microarray hybridisation. Additionally, PC concentrate has proved successful in the treatment of sepsis in non-randomised clinical trials (White *et al*, 2000a; Smith *et al*, 1997; Smith and White, 1999; de Kleijn *et al*, 2003) although a large scale randomised trial to compare these two agents has not been performed in severe sepsis patients. While both APC and PC have been successful in the treatment of severe sepsis in clinical trials (Smith *et al*, 1997; Smith and White, 1999; Bernard *et al*, 2001), any differences in the effect of APC as compared to PC on stimulated monocytes would be of interest in determining any advantage to the use of APC over PC in sepsis treatment. It would be useful to compare the large-scale gene profile results reported here to those from an identical experiment utilising APC. From the cytokine expression results obtained in this study, APC and PC both reduce LPS-induced monocyte activation. It appears that this is the primary effect of PC on activated THP-1 cells. Whether APC has further effects on activated monocytes, as seen with endothelial cells (Joyce *et al*, 2001), remains to be elucidated. However, it may be that PC is being activated upon addition to the monocyte cell culture thus resulting in similar effects by both APC and PC. Alternatively, PC may bring about its anti-inflammatory effects without the need for activation, whilst additional effects may only be possible when the protein is activated.

However, since APC appears to function as an anti-inflammatory agent *via* the EPCR (Taylor *et al*, 2000; Sturn *et al*, 2003) and EPCR binding enhances activation of PC to APC (Stearns-Kurosawa *et al*, 1996), the functional effects of PC in monocytes are most likely mediated by binding to EPCR and activation to APC. Hancock *et al* showed that the protein C receptor on monocytes binds APC but not PC or enzymatically inactive APC (Hancock *et al*, 1995), however, the EPCR is known to be expressed on monocytes (Galligan *et al*, 2001) and this receptor binds both APC and its zymogen PC with high affinity (Fukudome and Esmon, 1994). Both APC and PC decrease monocyte chemotaxis (Sturn *et al*, 2003), and this inhibition is blocked by the presence of anti-EPCR antibody, although a control EPCR antibody which does not block the APC/PC binding site did not affect the APC or PC ability to inhibit monocyte or neutrophil chemotaxis, indicating that ligation of APC or PC to its binding site on EPCR is necessary for the functional response to occur.

In healthy humans, there is a positive correlation between circulating PC and APC levels, supporting the hypothesis that under physiological conditions the conversion of PC to APC by thrombin-TM is dependent on circulating levels of PC. Thrombin formation changes the relationship between PC and APC levels, for example in healthy baboons, infusion of low levels of thrombin generate a proportional increase in APC levels (Hanson *et al*, 1993). In patients with severe sepsis, low protein C levels have been correlated with increased severity of thrombotic lesions and poor outcome (Powars *et al*, 1993; Brandtzaeg *et al*, 1989; Fijnvandraat *et al*, 1995). Faust *et al* reported that the level of endothelial TM and EPCR expression was lower in meningococcal patients than in controls, both in vessels with thrombosis and vessels without thrombosis (Faust *et al*, 2001). Skin biopsies were taken from the edge of purpuric or petechial lesions. They suggested that the reduction in endothelial expression of TM and EPCR results in impairment of PC activation, leading to the conclusion that administration of APC rather than PC would be more beneficial for severe sepsis patients (Faust *et al*, 2001; Esmon, 2000b; Yan and Dhainaut, 2001). However, it has been reported that treatment with PC concentrate is safe in patients with purpura fulminans and septic shock and leads to dose-related increases of plasma APC and resolution of coagulation balances (de Kleijn *et al*, 2003). However, there is still the argument that in some patients little increase in APC is achieved despite PC administration, and Faust *et al* reported that two patients in their study who received PC concentrate did not show detectable increases in their plasma APC levels despite increased plasma PC levels. Three different types of assay are used for determination of plasma PC

levels. An immunoassay methodology measures antigenic levels of PC, and activated partial thromboplastin time (APTT) and amiodolytic-based assays measure the functional activity of PC that has been activated with snake venom proteases. There is no commercial diagnostic kit available for the determination of APC levels in plasma. Several methods for measuring APC levels have been published, all of which involve many steps needing hours to weeks to perform (Yan and Dhainaut, 2001). Additionally, the assays, such as that used by Faust *et al*, measure only the free APC in a plasma sample, and do not take into account the APC in complex with serpins such as α-2 macroglobulin, α-1 antitrypsin, PCI, plasminogen activator inhibitor, and ceruloplasmin, and the APC bound to the EPCR. Furthermore, although Faust *et al* immunohistochemically showed a decrease in endothelial TM in skin biopsies taken from the edge of lesions in meningococcal sepsis patients, the mass of endothelial cells in other organs not affected by purpura fulminans where normal thrombomodulin levels exist, as shown in rat sepsis models (Laszik *et al*, 1994), was not taken into account, and the levels of TM on monocytes in sepsis patients was not investigated. Grey and Hancock demonstrated that expression of TM on monocytes is upregulated by LPS or TNF-α stimulation, in contrast to endothelial cells which show a decrease in surface TM (Grey and Hancock, 1996), and Gu *et al* reported that despite increasing EPCR shedding and sEPCR levels, endotoxin and thrombin also elevate EPCR mRNA levels in rats (Gu *et al*, 2000). The problem of increased sEPCR in the plasma sequestering free APC or PC would be improved by both PC or APC treatment. Thus levels of TM and EPCR in septic patients are likely to be high enough to allow normal conversion of protein C zymogen to APC and, taking into account the success of both APC and PC zymogen at treating purpura fulminans and meningococcal sepsis patients, it appears that both APC and PC are beneficial in the treatment of severe sepsis patients.

For this gene expression profile microarray experiment, we used RNA isolated from the THP-1 monocytic cell line. There are several advantages and disadvantages to using cell lines as opposed to using isolated primary human cells for such an experiment. Firstly, the use of primary monocytes has several disadvantages. RNA obtained from monocytes from just one individual can reflect a bias of the donor individual, and where a pool of RNA from several donors is used, it is difficult to ascertain the influence of individual effects, i.e. are the results representative of all monocytes or do they indicate the reaction of monocytes from just one or two individuals? For example, Rawadi *et al* looked at the production of IL-1β, TNF-α, and IL-6 in human monocytes from five donors stimulated

with LPS and heat inactivated *M. fermentans* (Rawadi and Roman-Roman, 1996). The responses of the different donors with regard to cytokine production were found to be quite varied, and therefore, due to this potential variability observed with human monocytes, a human cell line was used for their investigation. The primary monocyte population may also be contaminated by other leukocytes which may affect or change the monocyte response. Any primary monocyte isolation we perform is verified to be 95% pure by CD14 staining; however 100% purity can never be achieved. Primary monocytes are also difficult to maintain in culture due to their tendency to attach to cell culture containers, leading to difficulty in harvesting the cells for RNA isolation without damage or stimulation. They are also difficult to maintain in culture for long periods due to their need for certain growth factors. However, the advantage of the use of primary human monocytes is that they are a biologically relevant system. The advantage of a cell line such as THP-1 is that it grows in culture in suspension for many passages of division, mimics many of the secretory patterns of primary monocytes, and represents a system with low variation. However, the THP-1 cell line is cancerous, as most cell lines are, and it will therefore differ in some respects to primary human monocytes, as seen in the difference between THP-1s and primary monocytes in secretion levels of IL-6 in response to LPS. Not many studies have investigated the appropriateness of using the THP-1 cell line as model for primary monocytes. Heil *et al* found that the THP-1 cell line appeared to be a reasonable model for the relative ranking of toxicity of dental material components, but less predictive for assessing secretory response to these components, especially the reduction of LPS-induced TNF-α secretion (Heil *et al*, 2002). They concluded that primary monocytes and THP-1 cells show similar trends but that there are also distinct differences which demand that results with THP-1 be verified with primary monocytes. In our study, due to the difficulty of obtaining sufficient RNA from isolated human monocytes and the possibility of inter-individual variation in these cells, we chose to use the THP-1 cell line as a model system. However, we also verified some of the microarray results in primary human monocytes. Further study in this area would be enhanced by a similar gene profile experiment using primary human monocytes. This would both allow verification of the results obtained using THP-1 cells and also give further insight into the differences that may be seen when using this cell line as an alternative to primary cells.

As mentioned in the introduction of this chapter (section 3.1.2), the procoagulant protein thrombin can act as a pro-inflammatory agent, for example by enhancing LPS-induced inflammation (Hoffman and Cooper, 1995) or up-regulating ICAM-1 expression in

monocytes (Clark *et al*, 2003). Protein C is known primarily as an anticoagulant and its anti-thrombotic activity is derived from its ability to entirely inactivate FVa and FVIIIa, both critical for thrombin generation (Esmon, 1989). In order to ensure that the observed effects of APC on endothelial cells were independent of the ability of APC to inhibit thrombin generation, Joyce *et al* included hirudin, a potent thrombin inhibitor, in cell culture treatments for their microarray experiment, and found no effect of this inhibitor on the anti-inflammatory effects of APC (Joyce *et al*, 2001). We carried out a similar experiment to check for an influence by contaminating levels of thrombin in the cell culture and demonstrated that addition of hirudin to the cell culture had no effect on the ability of APC or PC to down-regulate LPS-induced TNF-α levels in monocytes.

In order to determine if the anti-inflammatory effects of APC and PC depend on incubation of the cells with APC or PC prior to LPS addition, we carried a out a time course experiment where addition of APC or PC was performed at various time points relative to LPS addition. The results demonstrated that addition of APC or PC up to thirty minutes after LPS addition still causes TNF-α downregulation in LPS treated cells. However, APC or PC addition sixty minutes or more after LPS stimulation does not cause TNF-α downregulation, possibly due to the sixty minutes of incubation with LPS alone, during which time the monocytes are activated and secrete TNF-α. Any effect of APC or PC addition at this stage is perhaps masked by the TNF-α already secreted into the medium. Alternatively, once the monocyte has been stimulated for a certain amount of time, APC or PC may not be effective at reducing this cell activation, requiring early addition in order to obtain maximal anti-inflammatory effects.

Protein C (PC) plasma concentration is 60-70 nM (3-5 μg/ml) in normal individuals (Esmon, 1987) whilst APC circulates at a concentration of approximately 40pM (1-3 ng/ml) (Gruber and Griffin, 1992). We utilised varying amounts of APC and PC in our experiments ranging from 200 μg/ml to 10 μg/ml (50 U/ml to 2.5 U/ml). One unit of PC refers to the amount of PC in 1 ml pooled normal plasma. Bernard *et al* administered recombinant APC at 24 μg/kg body weight per hour, which corresponds approximately to 0.348 μg/ml/hour for an average body weight of 72.5 kg containing 5 litres of blood (Bernard *et al*, 2001). In a clinical trial of PC concentrate (Baxter Hyland Immuno, Vienna, Austria), the same PC concentrate used in our experiments, 110 IU/kg (400μg) was initially administered followed by 10 IU/kg/hour (approximately 2 U/ml over the initial two to four hours). The dose was then adjusted on a daily basis with the aim of maintaining

plasma PC levels (White *et al*, 2000a). The dose administered by White *et al* is similar to the lowest PC concentration used in our experiments. This amount is just 2-fold the PC concentration in normal plasma. Different APC preparations can have differing functional activities as measured by APTT or amidolytic based assay (Hooper *et al*, 1998). Thus a preparation with a lower functional activity will require higher dosage concentrations in order to have similar effects as other preparations with higher activity.

Severe sepsis and septic shock remain important clinical problems and until recently no single effective therapy existed as treatment. Activated protein C is now known to have anti-inflammatory properties along with its well documented anticoagulant activity. These two properties are clearly important in the reduced morbidity and mortality seen in clinical trials of APC and PC in sepsis. Through gene profile studies, we showed that LPS treatment of monocytes causes the upregulation of a range of genes involved in chemotaxis, inflammation, coagulation, cellular proliferation and differentiation, transcriptional activation, signal transduction, and cell survival. Expression of all LPS induced genes was decreased in cells treated with PC prior to LPS administration. PC treatment of unstimulated cells induced few genes, leading to the conclusion that the principal effect of PC on monocytes is the attenuation of the cellular response to inflammatory stimulus, although the mechanism of this attenuation remains to be fully elucidated.

CHAPTER 4

Effect of Protein C and Heparin on Monocyte Toll-Like Receptor 4 and CD14 Levels

4.1 INTRODUCTION

4.1.1 CD14

CD14 is a myeloid marker antigen. Membrane CD14 (mCD14) is expressed on the surface of myeloid cells *via* a glycosylphosphatidyinositol (GPI) tail which attaches the protein to the cell membrane without a transmembrane segment (Simmons *et al*, 1989). A soluble form of the protein (sCD14) which is found free in plasma is thought to mediate the activation of epithelial and endothelial cells by LPS (Pugin *et al*, 1993). Overexpression of CD14 in mice renders the animals hypersensitive to LPS (Ferrero *et al*, 1993), whilst CD14-deficient mice are at least 10 times less sensitive to LPS than normal mice (Haziot *et al*, 1995). CD14 was known to play an essential role in LPS signalling for some years before the discovery of the Toll-like receptors. However, CD14 has no intracellular domain and so could not alone be responsible for LPS signalling in inflammatory cells.

4.1.2 TLR4

Toll-like receptor 4 was the first characterised mammalian TLR (Medzhitov *et al*, 1997). When over-expressed, it was shown to activate NF-κB and induce co-stimulatory molecules. TLR4 was discovered to be the signal transducing receptor for LPS when the endotoxin-unresponsiveness of C3H/HeJ and C57BL/10ScCr mouse strains, which exhibit natural tolerance to otherwise lethal doses of LPS, was found to be due to mutations in the Toll-like receptor 4 locus (*Tlr4*) (Poltorak *et al*, 1998; Qureshi *et al*, 1999). This discovery was confirmed in *Tlr4* knockout mice (Hoshino *et al*, 1999). The importance of TLR4 in humans was highlighted by Smirnova *et al* who identified an excess of rare TLR4 coding variants in meningococcal sepsis patients compared to controls (Smirnova *et al*, 2003). For efficient LPS recognition, TLR4 requires an additional molecule, MD-2, to complex with its extracellular domain (Shimazu *et al*, 1999).

4.1.3 LPS Activation Cluster

LPS initially complexes with LBP in the bloodstream, (Schumann *et al*, 1990) and the formation of the LBP-LPS complex facilitates delivery of LPS to the CD14 receptor on the monocyte cell surface (Wright *et al*, 1990). The main function of CD14 is thought to be to catalyse the transfer of LPS from the bloodstream to the cell membrane where it associates with a complex of receptors. Thus CD14 initially binds LPS and transfers it to the TLR4-MD-2 complex (da Silva Correia *et al*, 2001). An activation cluster, formed after LPS stimulation and composed of CD14, TLR4, CD11b/CD18, CD55, Fcγ RIIIa (CD16a), CD36, CD81, and Fcγ receptors CD32 and CD64, has been described (Pfeiffer *et al*, 2001), indicating that CD14 and TLR4 alone are not solely responsible for LPS recognition.

4.1.4 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a significant health problem in the developed world, affecting 1 in 1000 individuals in western countries. Patients with IBD suffer from chronic inflammation in the colon affecting the large and/or small intestine. Symptoms include diarrhoea, abdominal pain, fever, rectal bleeding, weight loss, and nausea. In severe cases, death can occur as a result of malnutrition, dehydration, and anaemia. IBD is thought to be caused by interacting genetic and environmental factors (Sartor, 1997). Clinical behaviour of the disease is very variable. In most theories of IBD aetiology, either pathogenic or lumenal bacteria stimulate the mucosal and systemic immune systems to activate the inflammatory cascade, usually by breaching the intestinal mucosal barrier and exposing lamina propria immune cells. Macrophages and T lymphocytes, once stimulated by the initial immune response, secrete cytokines which then attract other immune cells to the mucosal wall, continuing the inflammatory process. IBD is associated with increased levels of pro-inflammatory cytokines, chemokines, and adhesion molecules, and therefore has been defined as an immune mediated disorder. Additionally, NF-κB, a pivotal transcription factor in the inflammatory cascade, has been implicated in intestinal inflammation (Schreiber *et al*, 1998). However, IBD is also associated with increased activation of the coagulation pathway and an increased risk of thromboembolic complications. Microthrombi have been identified in intestinal biopsies from patients with IBD (Talbot *et al*, 1986; Dhillon *et al*, 1992), indicating that the disease may be associated with a hypercoagulable state.

4.1.5 Low Molecular Weight Heparin for IBD Treatment

The current treatment of choice for patients with moderate to severely active IBD involves immunosuppression with high dose steroids. Anti-TNF therapy has also been used in Crohn's disease (CD), with varying success. More recently, studies have demonstrated that heparin without concomitant steroids may be an alternative first line treatment in active IBD (Ang *et al*, 2000; Torkvist *et al*, 1999). The mode of action of heparin in these patients is not fully understood. Heparin has long been known for its anticoagulant effects, however it also has anti-inflammatory properties. Salas *et al* demonstrated that heparin attenuates TNF-α induced inflammatory response through a CD11b dependent mechanism (Salas *et al*, 2000). At the cellular and intracellular level a link between the inflammatory and coagulation cascades may explain the mechanism of action of heparin in IBD and in doing so may further explain the aetiology of IBD.

4.1.6 TLR4 in IBD

IBD is thought to be a result of a dysfunction in the control of mucosal immune responses. Although both CD and ulcerative colitis (UC), two forms of IBD, have differing clinical manifestations, an immune response to intestinal bacteria though TLRs is thought to be the critical factor the development of both these diseases (French and Pettersson, 2000). Cario *et al* demonstrated the expression of a number of TLRs on intestinal epithelial cells (IECs) (Cario *et al*, 2000) indicating that TLRs may be important in detection and monitoring of lumenal bacteria, with significant infiltration of monocytes and macrophages only when dysfunction or infection occurs. Moreover, IECs of patients with both UC and CD show strong upregulation of TLR4 compared to IECs from healthy humans (Cario and Podolsky, 2000), and increased levels of TLR2 and TLR4 in inflamed intestinal mucosa, localised in intestinal macrophages, has also been demonstrated (Hausmann *et al*, 2002). Furthermore, decreased expression of IEC TLR4 protects against dysregulated proinflammatory gene expression in response to LPS (Abreu *et al*, 2001). An increase in expression and activation of TLR4 by IECs would lead to recruitment of monocytes/macrophages to the site of inflammation by way of increased secretion of TNF-α and IL-8, leading to further synthesis of pro-inflammatory cytokines such as TNF-α, IL-6 and IFN-γ, and more severe disease.

4.1.7 TLR4 and CD14 in Sepsis

The role of TLR4 as the primary LPS receptor has been well established. It therefore follows that the levels of TLR4 on the cell surface may determine the extent of the cell response to LPS activation. The same may apply to levels of CD14 on the monocyte cell surface where it acts as the initial LPS receptor before transferring it to the LPS activation cluster which includes TLR4. Tissue TLR4 levels during sepsis have been studied, mainly in mouse models. Williams *et al* found that liver, lung, and spleen TLR4 mRNA and protein levels are increased in sepsis-induced mice compared to controls and that early increases in TLR4 expression correlated with mortality whilst TLR4 gene blunting correlated with long-term survival (Williams *et al*, 2003). Bihl *et al* showed that transgenic mice over-expressing TLR4 responded more to LPS than mice with fewer copies of the gene, although the mice with more gene copies survived longer and in a greater percentage when infected by *Salmonella typhimurium*. However, the early improvement in disease resistance was not seen later during infection as those mice over-expressing TLR4 developed an excessive inflammatory response that proved detrimental to the host (Bihl *et al*, 2003). LPS induces TLR4 surface expression on human monocytes and granulocytes, and anti-TLR4 antibodies inhibit LPS mediated TNF-α induction by approximately 70%, whilst anti-CD14 inhibit TNF-α induction nearly completely, indicating the essential nature of these receptors for cellular activation by LPS (Mirlashari and Lyberg, 2003). Bosisio *et al* showed that IFN-γ upregulates both protein and mRNA levels of TLR4 in human monocytes, however, they found that although LPS upregulated TLR4 mRNA levels it downregulated protein levels of the receptor on the cell surface (Bosisio *et al*, 2002). In a mouse model of sepsis CD14 mRNA levels are induced in the lung, kidney, and liver tissues (Wang *et al*, 1998). Human monocytes incubated with LPS-LBP complex increase CD14 expression, although CD14 expression on monocytes from septic patients is lower than on monocytes from normal controls (Birkenmaier *et al*, 1992). In human monocytes, LPS treatment has been found to increase the levels of cell surface CD14 after 30 minutes (Jiang *et al*, 2000), with a peak at 1 to 3 hours (Marchant *et al*, 1992), although after 8 hours CD14 levels have been reported to be downregulated in response to LPS treatment (Grey *et al*, 1994). Overall, it appears that levels of TLR4 and CD14 are increased in response to LPS and this increase, although beneficial in the short term in responding to bacterial infection, can lead to an excessive inflammatory response that perpetuates the immune cell activation which plays a driving role in the pathophysiology of severe sepsis.

4.1.8 Aims

The purpose of this study was to investigate two agents, protein C and low molecular weight heparin, that are effective in the treatment of meningococcal sepsis and IBD respectively (Smith *et al*, 1997; Ang *et al*, 2000; Torkvist *et al*, 1999). Both conditions are associated with increased coagulation and inflammation, processes which are thought to be integrally linked in the pathophysiology of these diseases. The mechanism of therapeutic action of either PC or LMWH is unclear. Both have well known anti-coagulant properties but also have anti-inflammatory actions. Whether both of these properties are of equal importance in the treatment of these diseases, or whether the anti-inflammatory or the anti-coagulant effect is more prominent, is yet to be determined.

TLR4 and CD14 are the principal receptors for LPS on the monocyte cell surface, and TLR4 mediates the signal that activates monocytes in response to LPS binding. Therefore a downregulation of levels or blocking of these receptors leads to a decrease in cell activation which would be beneficial in conditions such as IBD or septicaemia which are associated with a predominantly pro-inflammatory profile (Williams *et al*, 2003; Abreu *et al*, 2001). We therefore investigated the effect of PC and LMWH on cell surface TLR4 and CD14 levels, and on the gene expression of TLR4, as these two molecules are key in the activation of monocytic cell responses to LPS. Downregulation of LPS receptors is a potential mode of action for agents such as protein C that are capable of blocking the LPS-induced NF-κB induction of gene expression and attenuating cell inflammatory responses. Moreover, the binding of heparin to neutrophils through a CD11b dependent mechanism potentially indicates anti-inflammatory action of heparin though disruption of the LPS activation cluster.

4.2 MATERIALS AND METHODS

4.2.1 Cell Viability and Proliferation

Quantitation of cell death and cell lysis was based on the measurement of lactate dehydrogenase (LDH) activity in the supernatant. LDH is released from the cytosol of damaged cells into the supernatant. The Cytotoxicity Detection Kit (Roche Molecular Biochemicals, Basel, Switzerland) was used according to the manufacturer's instructions. Quantitation of cell proliferation was based on the cleavage of the tetrazolium salt WST-1 (water soluble tetrazolium salt) by mitochondrial dehydrogenases in viable cells. Measurement of cell proliferation using the Cell Proliferation Reagent WST-1 (Roche Molecular Biochemicals) was carried out according to the manufacturer's instructions. Both tests included negative controls consisting of medium and the test substance (LPS, APC/PC, or LMWH) but no cells. Positive controls, consisting of LDH (0.5 U/ml) and each test substance, were also used in the cytotoxicity detection test. Cell viability and proliferation was measured in both THP1 cells and primary human monocytes under a variety of conditions as set out in Table 4.1. The untreated sample was the low control in the cytotoxicity detection test and the high control in the cell proliferation test. A cell sample treated with 2 % Triton x100 was the high control in the cytotoxicity detection test and the low control in the cell proliferation test.

Table 4.1 Treatment conditions for cell viability and cell proliferation assays. Both THP-1 cells and primary monocytes were tested for 4 hour and 24 hour treatment times.

Conditions
Untreated
LPS (10 μg/ml)
LPS (100 ng/ml)
LPS (10 μg/ml) + APC (200 μg/ml)
LPS (10 μg/ml) + APC (100 μg/ml)
LPS (10 μg/ml) + APC (25 μg/ml)
APC (200 μg/ml)
APC (100 μg/ml)
APC (25 μg/ml)
LPS (10 μg/ml) + PC (200 μg/ml)
LPS (10 μg/ml) + PC (100 μg/ml)
LPS (10 μg/ml) + PC (25 μg/ml)
PC (200 μg/ml)
PC (100 μg/ml)
PC (25 μg/ml)
LPS (10 μg/ml) + LMWH (1000 U/ml)
LPS (10 μg/ml) + LMWH (500 U/ml)
LPS (10 μg/ml) + LMWH (100 U/ml)
LMWH (1000 U/ml)
LMWH (500 U/ml)
LMWH (100 U/ml)

4.2.2 Real-Time PCR to Quantify TLR4 Gene Expression

Tissue culture and Primary Cell Treatment Conditions

THP-1 cells (cell culture procedures Section 2.2.1) or primary human monocytes (isolation procedure Section 2.2.2) were counted and assessed for viability by staining with 0.5% Trypan blue (Sigma) and resuspended at 1 x 10^6 cells/ml. Cells were seeded into 24-well microtitre plates in 1 ml aliquots. These were then stimulated with appropriate concentrations of LPS (10 ng/ml or 10 μg/ml), IFN-γ (500 IU/ml) (R&D Biosystems), APC (10-200μg/ml), PC (10-200μg/ml), and LMWH (2.5-100 IU/ml)(Tinzaparin, Leo laboratories, Dublin, Ireland) and incubated for relevant times (90 minutes to 24 hours).

Preparation of cDNA

Each RNA sample, 4 μg, was reverse transcribed in a total reaction volume of 30 μl, as described in Section 2.2.6. As controls, 'no amplification' and 'no template' samples were also prepared by omitting AMV reverse transcriptase or template RNA respectively.

Real-Time PCR Analysis of the TLR4 Gene Expression Using TaqMan Technology

Relative mRNA levels of TLR4 in monocytes were investigated with the use of TaqMan technology. The area of the TLR4 gene spanned by the primers was amplified using AmpliTaq Gold™ DNA polymerase and the ABI PRISM® 7700 Sequence Detection System. A probe, fluorescently labelled with FAM reporter dye at the 5' end and TAMRA quencher at the 3' end, was designed be specific to the TLR4 gene and to anneal between the forward and reverse primers. During the PCR reaction, the 5' nuclease activity of the DNA polymerase cleaves the probe hybridised to the target sequence, thus separating the reporter dye from the quencher dye, and resulting in increased fluorescent emission. The detection of this fluorescence is used to quantify relative concentration of the target gene sequence.

Primers and probes specific to the TLR4 gene were designed using the Primer Express® Oligo Design software. Details on the use of this software can be found at http://www.appliedbiosystems.com/support/tutorials/#. The location of primers and probes in the TLR4 gene are shown in Figure 4.1

```
421 tctacaaaat ccccgacaac ctccccttct caaccaagaa cctggacctg agctttaatc
        FORWARD PRIMER                              PROBE
481 ccctgaggca tttaggcagc tatagcttct tcagtttccc agaactgcag gtgctggatt
      EXON 3/4 BOUNDARY
541 tatccag|gtg tgaaatccag acaattgaag atggggcata tcagagccta agccacctct
       REVERSE PRIMER
601 ctaccttaat attgacagga aaccccatcc agagtttagc cctgggagcc ttttctggac
```

Figure 4.1 Primer and probe sequence for real-time PCR amplification and relative quantitation of TLR4 gene expression. The sequence shown corresponds to the published TLR4 gene mRNA sequence (NM_003266). The reverse primer was designed to span the exon 3/exon 4 boundary. See Appendix C for TLR4 gene complete coding sequence (AF177765) and complete mRNA sequence.

The probe sequence selected was designed to hybridise to the TLR4 gene in exon 3. The primers were selected to be as close as possible to the probe sequence without overlapping it, and thus to amplify an 80 base pair fragment of DNA. Sequences of both primers and probes were selected to have a G/C content between 40% and 55%, and primers were selected to have a melting temperature (T_m) between 57 and 63°C, with no more than a 2°C difference between the T_m of the two primers. Runs of four or more Gs were avoided, as were sequences that would cause secondary structure such as hairpin loops. The probe was designed to have a T_m approximately 10°C higher than those of the primers. Real-time PCR primers and probe (FAM-labelled and TAMRA quenched) characteristics are shown in Table 4.2.

Table 4.2 Real-time PCR primer and probe sequences for relative quantitation of the TLR4 gene expression.

Oligonucleotide	Sequence	G/C content	T_m
TLR4 forward primer	5'-CCTGAGGCATTTAGGCAGCTAT -3'	50%	61.8°C
TLR4 reverse primer	5'-TCTGGATTTCACACCTGGATAAATC-3'	40%	62.4°C
TLR4 probe	5'-AGCACCTGCAGTTCTGGGAAACTGAAGA-3'	50%	71.7°C

Primer and probe concentrations were optimised in order to determine the minimum concentration of each required for maximum fluorescence emission. To optimise primers concentrations, a series of PCR reactions was performed using varying concentrations of both forward and reverse primers, as detailed in the matrix below.

	Forward primer 50 nM	**Forward primer 300 nM**	**Forward primer 900 nM**
Reverse primer 50 nM	50/50	300/50	900/50
Reverse primer 300 nM	50/300	300/300	900/300
Reverse primer 900 nM	50/900	300/900	900/900

A 25 µl reaction was set up in triplicate for each combination of primer concentrations. In each reaction, 2 µl cDNA (250 ng), prepared from untreated human monocytes, was used as the target, along with 12.5 µl TaqMan Universal PCR Master Mix (Applied Biosystems), sense and antisense primer (volume varies depending on 50, 300, or 900 nM final concentration needed), TLR4 specific probe (to a final concentration of 200 nM), and nuclease free H_2O water (to reach the final reaction volume of 25 µl). Reaction mixes, along with NTC and NAC samples, were aliquoted into a GeneAmp 96 well plate (Applied Biosystems), sealed with an ABI PRISM™ Optical Adhesive Cover (Applied Biosystems), and loaded for real-time PCR analysis on the 7700 Sequence Detection System. Thermal cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The probe concentration was optimised by running triplicate reactions containing varying probe concentrations (25 to 225nM) and the primer concentrations obtained from the primer optimisation analysis. The remaining components of the reaction mix and the cycling parameters were the same as those used for the primer optimisation, and NTC and NAC samples were also included.

To select between standard curve or comparative C_T methods, template titration standard curves were prepared for both target and control genes in separate tubes. The general protocol for this is outlined in Section 3.2.6. A 2-fold serial dilution of LPS-treated human monocyte cDNA was prepared (from x 1 to x 0.0625). The reaction mix, set up in triplicate, consisted of 2 µl of the appropriate target cDNA concentration, 12.5 µl TaqMan Universal PCR Master Mix, 200 nM final concentration TLR4 gene specific probe, 300 nM final concentration of both sense and antisense primer, or 1.25 µl endogenous control MGB PDAR, made up to a 25 µl final volume with nuclease free H_2O. Cycling conditions were as detailed above for the primer optimisation. As the difference between the target and control standard curve slope was greater than 0.1 the standard curve method was utilised for relative quantitation of TLR4 gene expression, using the reaction mix as listed above and the cycling conditions used for the primer optimisation. Relative concentrations were calculated from the standard curve using the equation [(sample C_T value – b)/m] where m is the slope of the line, and b is the y-intercept. The relative amount of target was divided by the relative amount of endogenous control in order to obtain the normalised amount of target expression. Generally, the cDNA sample with the lowest gene expression was designated as the calibrator for comparison of gene expression levels.

4.2.3 Flow Cytometry to Quantify TLR4 and CD14 Cell Surface Expression

Blood Treatment Conditions

In a 96 well tissue culture plate (Iwaki), 100 µl of citrate-anticoagulated whole blood was aliquoted per well. Selected blood samples were treated with APC (50 µg/ml or 20 µg/ml) or PC (50 µg/ml or 20 µg/ml) for 30 minutes at 37°C, or with LMWH (1000, 100, or 10 IU/ml) for 12 minutes at 37°C. LPS (1 ng/ml) was then added to appropriate wells and samples were incubated at 37°C for 4 or 24 hours prior to fluorescent labelling for FACS analysis. Samples were set up either in duplicate, and the experiment repeated, or in triplicate. Treatment samples for each blood sample are detailed in Table 4.3.

Table 4.3 Treatment conditions for whole blood samples prior to analysis of TLR4 and CD14 cell surface detection.

Sample	Treatment	Time
Whole blood	No treatment	4 and 24 hours
Whole blood	LPS (1 ng/ml)	4 and 24 hours
Whole blood	PC (50 µg/ml) + LPS	4 and 24 hours
Whole blood	PC (20 µg/ml) + LPS	4 and 24 hours
Whole blood	APC (50 µg/ml) + LPS	4 and 24 hours
Whole blood	APC (20 µg/ml) + LPS	4 and 24 hours
Whole blood	PC (20 µg/ml)	4 and 24 hours
Whole blood	APC (20 µg/ml)	4 and 24 hours
Whole blood	No treatment	4 and 24 hours
Whole blood	LPS (1 ng/ml)	4 and 24 hours
Whole blood	LMWH (1000 IU/ml) + LPS	4 and 24 hours
Whole blood	LMWH (100 IU/ml) + LPS	4 and 24 hours
Whole blood	LMWH (10 IU/ml) + LPS	4 and 24 hours
Whole blood	LMWH (100 IU/ml)	4 and 24 hours

Staining Protocol and FACS Analysis of Monocytes in Whole Blood

Two different fluorescent staining protocols were used to investigate the cell surface levels of TLR4 and CD14. The first method involved the direct staining of human primary monocytes in whole human blood (Holmes *et al*, 2002). In 1.5 ml tubes, 50 µl citrate-anticoagulated whole blood samples were labelled with 1 µl anti-human $CD14^{RPE}$ mAb (Dako, Glostrup, Denmark) and 5 µl anti-human $TLR4^{FITC}$ mAb (Abcam, Cambridge, UK) in triplicate. As negative isotype controls, 2.5 µl FITC-labelled IgG_{2a} and 0.6 µl RPE-labelled IgG_{2a} were used to label separate 50 µl samples of whole blood. Blood samples were gently vortexed and incubated in dark at 4°C for 30 minutes. Red cells were lysed by

adding 1 ml of FACS lysing solution (Becton Dickinson), gently vortexing, and incubating for 10 minutes in the dark at room temperature. Samples were centrifuged at 1000 x g for 5 minutes (brakes off), the supernatant was removed and the pellet was washed twice with 1 ml CellWASH (Becton Dickinson) before being fixed with 0.5 ml CellFIX (Becton Dickinson) and analysed within 48 hours. Flow cytometric acquisition and analysis of 1000 monocytes was performed on a FACsCalibur flow cytometer (Becton Dickinson), where monocytes were defined as CD14 positive cells. The fluorescent intensity of the RPE-labelled (CD14) antibody was detected on the fluorescence-2 channel, whilst the FITC-labelled (TLR4) antibody was detected on the fluorescence-1 channel. Data analysis was performed using CellQuest software (Becton Dickinson).

Cell Culture Conditions

Primary human monocytes (isolation procedure Section 2.2.2) were counted, assessed for viability by staining with 0.5% Trypan blue (Sigma), and resuspended at 1 x 10^6 cells/ml. Cells were seeded into 96-well microtitre plates in 100 µl aliquots. These were then stimulated with appropriate concentrations of LPS (10 µg/ml) and APC (25-200µg/ml) and incubated 4 or 20 hours.

Staining Protocol and FACS Analysis of Isolated Primary Human Monocytes

The second method used to investigate the cell surface levels of TLR4 and CD14 involved the direct staining of isolated human primary monocytes. Cells (generally 1 x 10^5/sample making a working concentration of 1x 10^6 cells/ml) were washed once with buffer (PBS with 1% BSA and 0.15% sodium azide, 0.2 µm filtered). In order to block Fc receptors and prevent non-specific binding of the CD14- and TLR4-specific antibodies, the primary human monocytes had been cultured in medium containing autologous serum which acts as an FcR blocking reagent. Cells were resuspended in 100 µl buffer and incubated with 1 µl r-phycoerythrin (RPE) conjugated mouse monoclonal antibody against human CD14 (anti-human $CD14^{RPE}$ mAb) (Dako, Glostrup, Denmark) and 5 µl fluorescein isothiocyanate (FITC) conjugated mouse monoclonal antibody against human TLR4 (anti-human $TLR4^{FITC}$ mAb) (Abcam, Cambridge, UK) for 30 minutes at 4°C in the dark. As negative controls, samples labelled with 0.6 µl RPE-conjugated isotype control antibody (IgG_{2a}) and labelled with 2.5 µl FITC-conjugated isotype control antibody (IgG_{2a}) (both Dako, Glostrup, Denmark) were also prepared. After the labelling step, cells were washed twice to remove any unbound antibody and resuspended in 700 µl buffer for analysis on a FACsCalibur flow cytometer (Becton Dickinson, Sunnyvale, CA, USA). Living cells were

gated on a dot plot display of forward scatter versus side scatter, and the fluorescent intensity of the RPE-labelled (CD14) antibody was detected on the fluorescence-2 channel, whilst the FITC-labelled (TLR4) antibody was detected on the fluorescence-1 channel. Data analysis was performed using CellQuest software (Becton Dickinson).

4.3 RESULTS

4.3.1 Cell Viability and Proliferation after LPS, APC, PC, or LWMH Treatment

Determination of the effect of treatment of LPS, APC, PC, or LMWH on primary human monocytes and THP1 cells for 4 or 24 hours was carried out by testing for cell viability using two different methods. The first measured cell death and cell lysis, the second measured cell proliferation. Results are shown in Figures 4.2 to 4.5. Figures 4.2 and 4.3 show that after 4 hours incubation, none of the conditions tested were cytotoxic to the cells which showed close to 100% proliferation under all conditions. However, after 24 hours incubation, the cells were much less viable, with 50% or less proliferation under the various treatments.

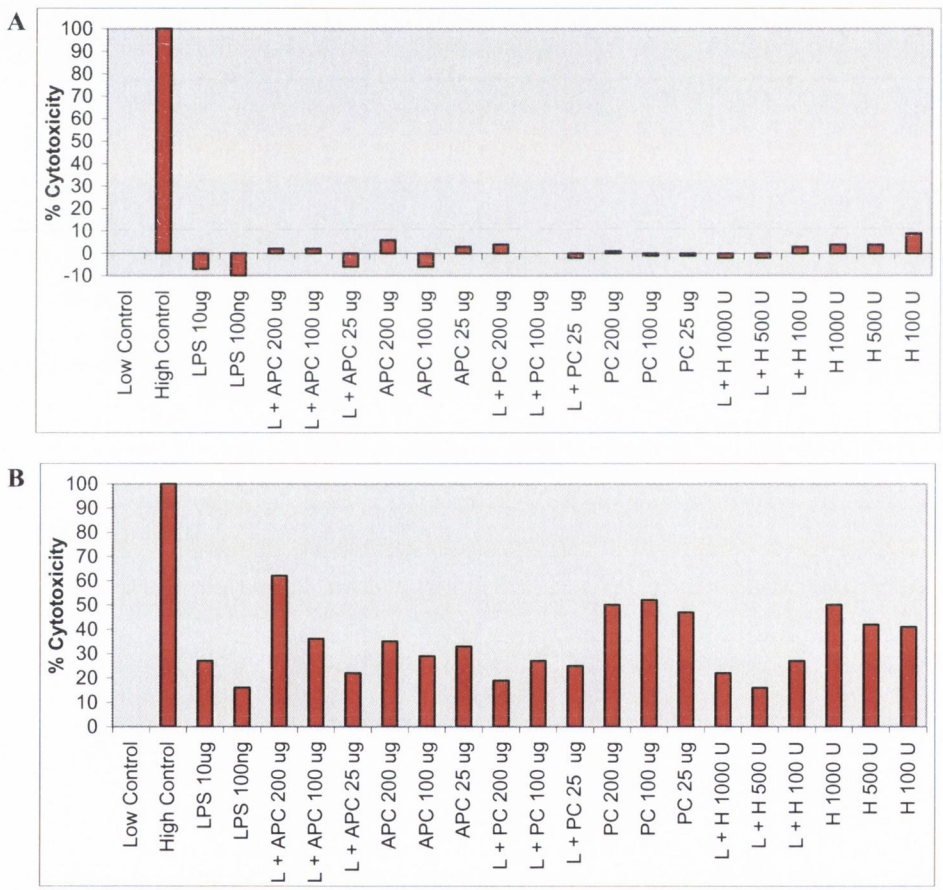

Figure 4.2 Quantitation of LPS, APC, PC, or LMWH (H) cytotoxicity on primary human monocytes by measurement of LDH release. Primary human monocytes (concentration 1 x 10^6/ml) were treated with the appropriate substance at the amount indicated (μg or ng for LPS and APC/PC, units (U) for LMWH) per million cells for (A) 4 hours and (B) 24 hours before LDH release measurement. The low control was an untreated sample of monocytes and a cell sample treated with 2% Triton x100 was used as the high control. L = LPS (10 μg/ml).

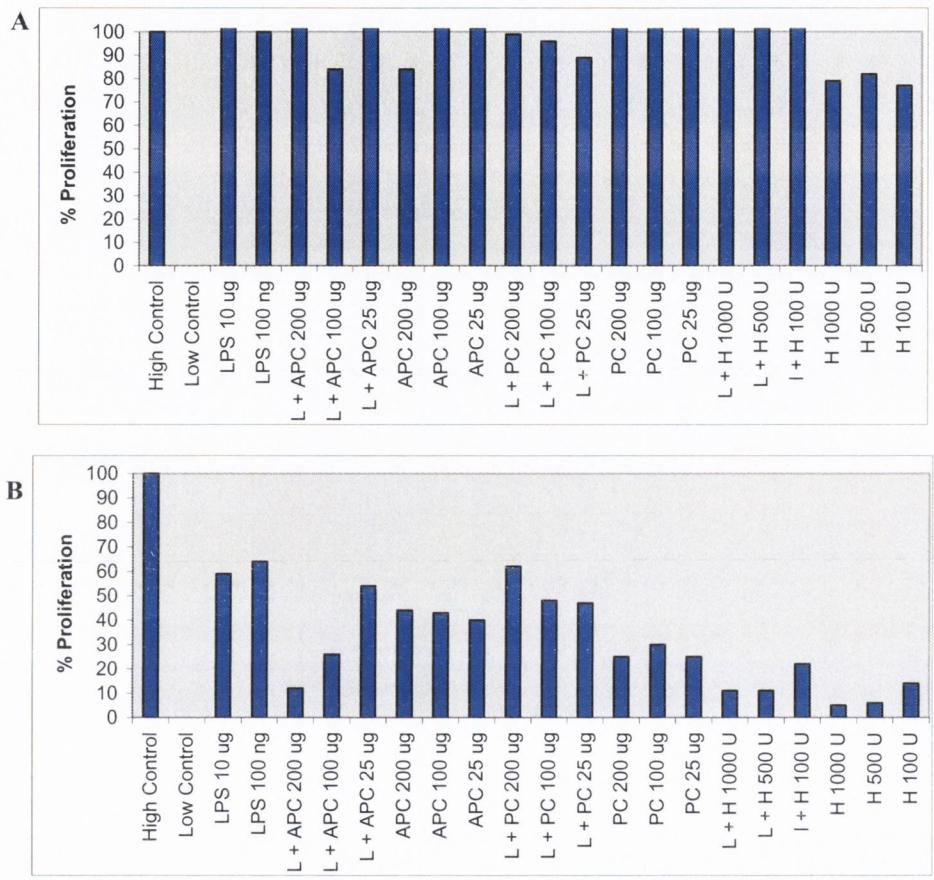

Figure 4.3 Quantitation of primary human monocyte cell proliferation after LPS, APC, PC, or LMWH (H) treatment by measurement of WST-1 cleavage by mitochondrial dehydrogenases in viable cells. Primary human monocytes (concentration 1 x 10^6/ml) were treated with the appropriate substance at the amount indicated (μg or ng for LPS and APC/PC, units (U) for LMWH) per million cells for (A) 4 hours and (B) 24 hours before measurement of WST-1 cleavage. The low control was a cell sample treated with 2% Triton x100 and the high control was an untreated sample of monocytes. L = LPS (10 μg/ml).

Figures 4.4 and 4.5 demonstrate that all treatment conditions were non-cytotoxic to THP-1 cells after both 4 and 24 hours incubation, with cell proliferation close to 100% in all cases.

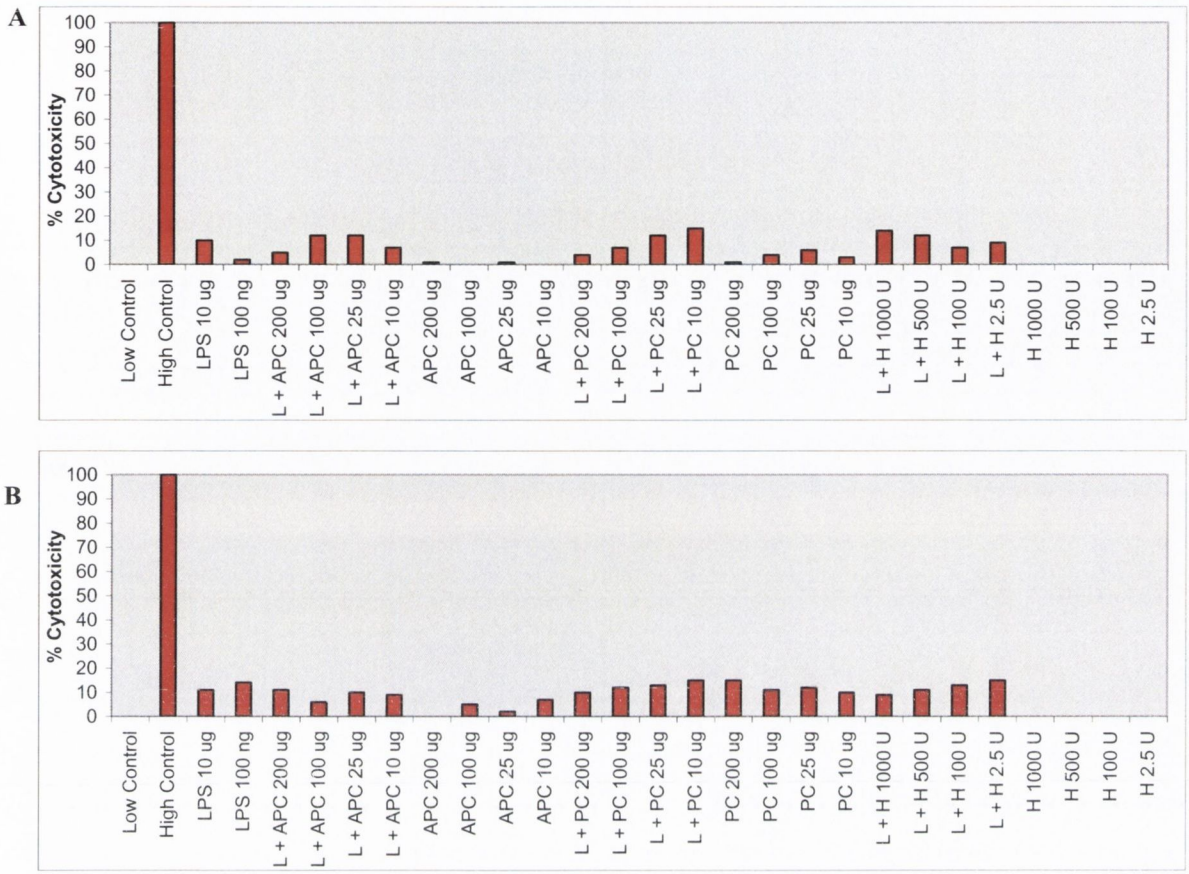

Figure 4.4 Quantitation of LPS, APC, PC, or LMWH (H) cytotoxicity on THP1 cells by measurement of LDH release. THP1 cells (concentration 1 x 10^6/ml) were treated with the appropriate substance at the amount indicated (µg or ng for LPS and APC/PC, units (U) for LMWH) per million cells for (A) 4 hours and (B) 24 hours before LDH release measurement. The low control was an untreated sample of cells and a cell sample treated with 2% Triton x100 was used as the high control. L = LPS (10 µg/ml).

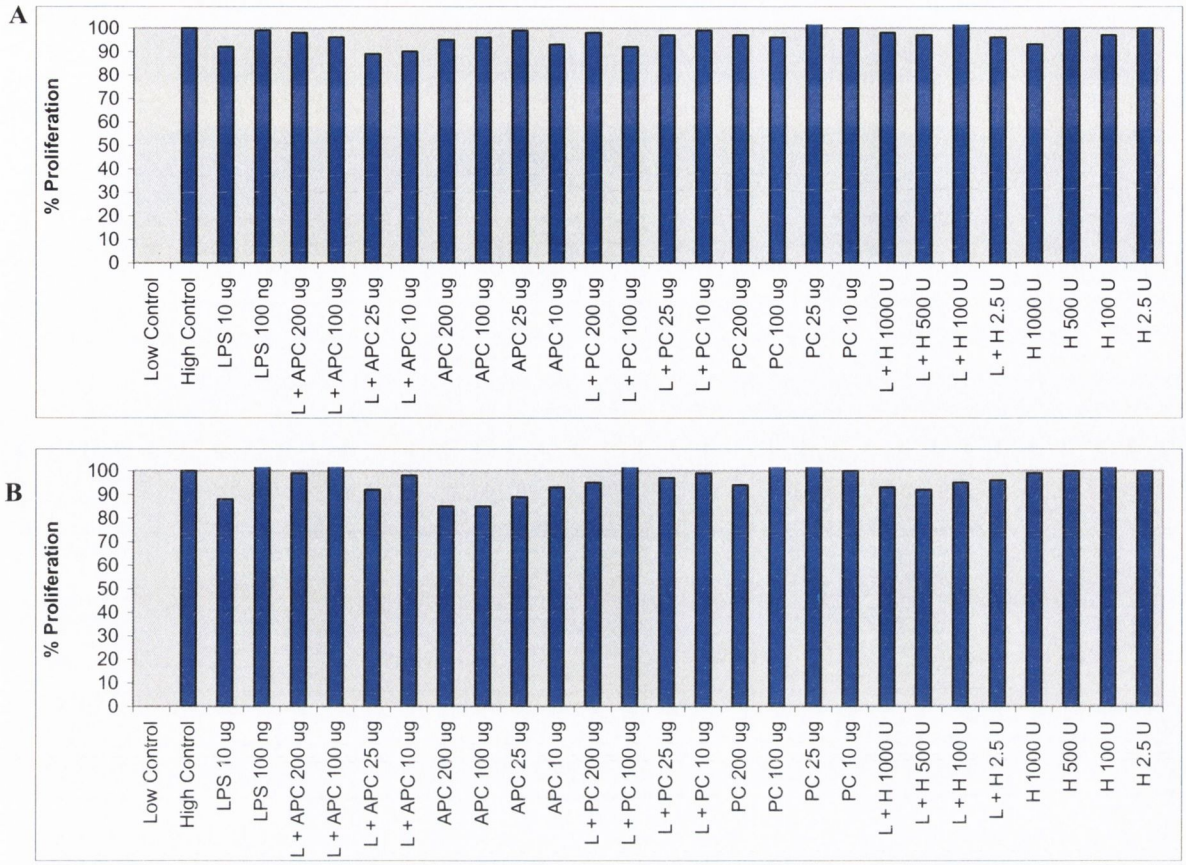

Figure 4.5 Quantitation of THP1 cell proliferation after LPS, APC, PC, or LMWH (H) treatment by measurement of WST-1 cleavage by mitochondrial dehydrogenases in viable cells. THP1s (concentration 1 x 10^6/ml) were treated with the appropriate substance at the amount indicated (µg or ng for LPS and APC/PC, units (U) for LMWH) per million cells for (A) 4 hours and (B) 24 hours before measurement of WST-1 cleavage. The low control was a cell sample treated with 2% Triton x100 and the high control was an untreated sample of monocytes. L = LPS (10 µg/ml).

4.3.2 Real Time PCR to Quantify Relative Toll-Like Receptor 4 Gene Expression

TLR4 Real Time PCR Assay Optimisation

Relative quantitation of TLR4 mRNA levels was carried out by real-time PCR and TaqMan technology (Section 4.2.2). Primer and probe oligonucleotides specific to the TLR4 gene were designed and used to perform the PCR amplification and real-time analysis. Primer concentrations were optimised prior to relative quantitation of the TLR4 gene itself in order to determine the minimum concentration needed for analysis by carrying out a series of amplification reactions using different concentrations of the forward and reverse primers. The resulting C_T values (the cycle at which a statistically significant increase in ΔR_n is first detected) are shown in Figure 4.6, and the logarithmic amplification plot of ΔR_n (background-normalised fluorescence emission intensity) against cycle number is shown in Figure 4.7. From the results obtained (Figure 4.6), 300 nM forward primer and 300 nM reverse primer were chosen as the optimal concentrations for this assay.

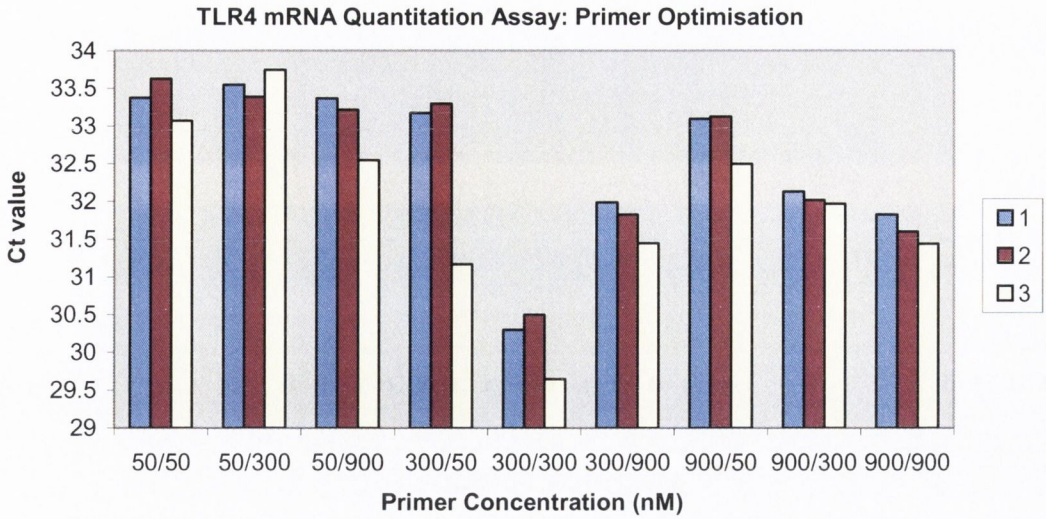

Figure 4.6 The effect of varying primer concentrations on TLR4 gene real-time PCR amplification. Triplicate assays are displayed (1, 2, and 3). Forward/reverse primer concentrations are on the x-axis.

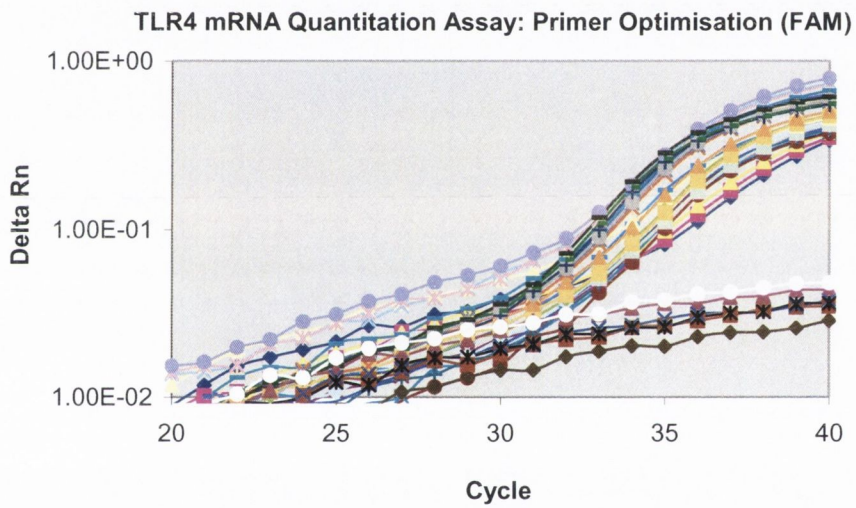

Figure 4.7 Logarithmic amplification plot of varying primer concentrations for TLR4 gene real-time PCR amplification. ΔR_n (background-normalised fluorescence emission intensity) is shown on the y-axis.

Subsequently, probe concentration was optimised using the previously optimised primer concentrations (300 nM/300 nM) and varying concentrations of probe (25 to 225 nM) in a real-time amplification of the TLR4 gene segment. The resulting C_T values are shown in Figure 4.8, and the logarithmic amplification plot of ΔR_n against cycle number is shown in Figure 4.9. From the results detailed in Figure 4.8, a probe concentration of 150 nM was chosen as the lowest probe concentration to give optimal fluorescent emission values.

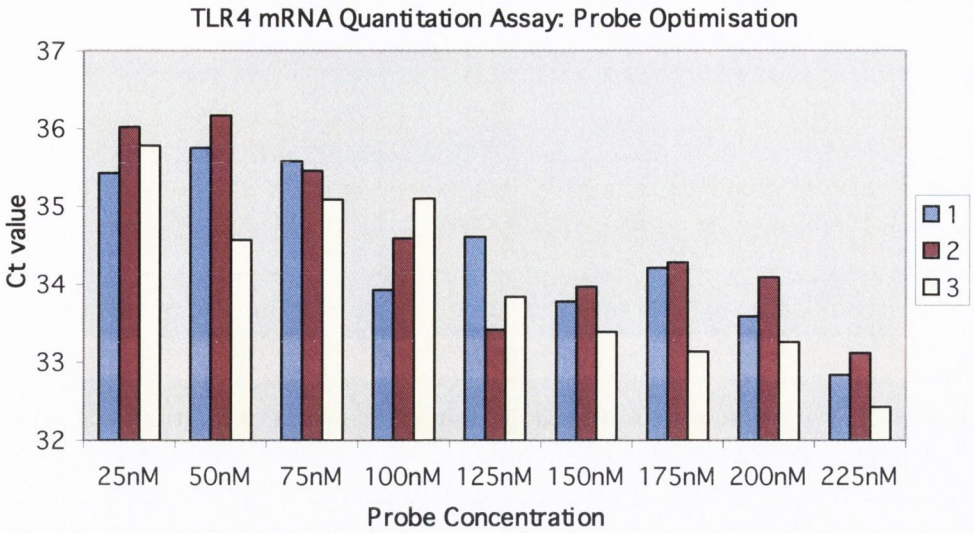

Figure 4.8 The effect of varying probe concentrations on TLR4 gene real-time PCR amplification. Triplicate assays are displayed (1, 2, and 3).

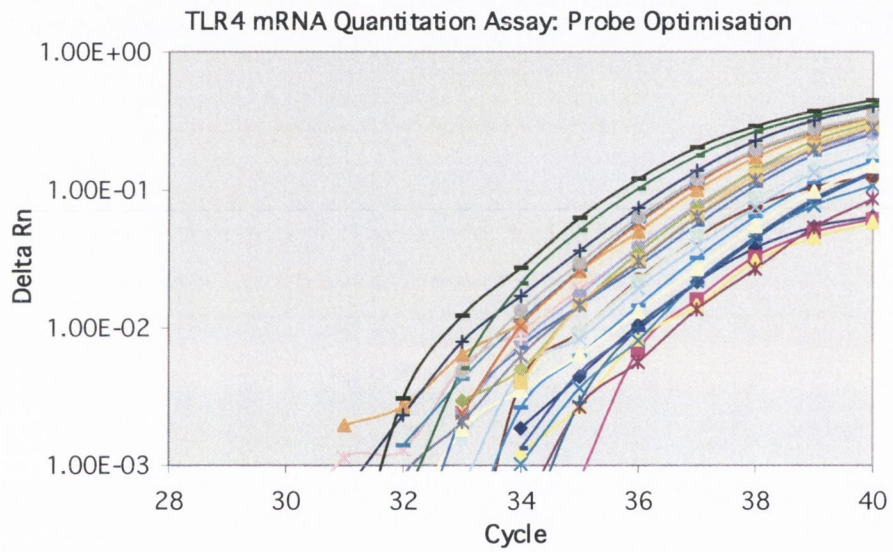

Figure 4.9 Logarithmic amplification plot of varying probe concentrations for TLR4 gene real-time PCR amplification. ΔR_n (background-normalised fluorescence emission intensity) is shown on the y-axis.

Finally, the standard curve method was chosen as the appropriate method for relative quantitation of TLR4 gene expression as it was found that the slopes of the TLR4 (target), 18S rRNA (endogenous control), and GAPDH (endogenous control) standard curves differed by more than 0.1. The standard curves are shown in Figure 4.10.

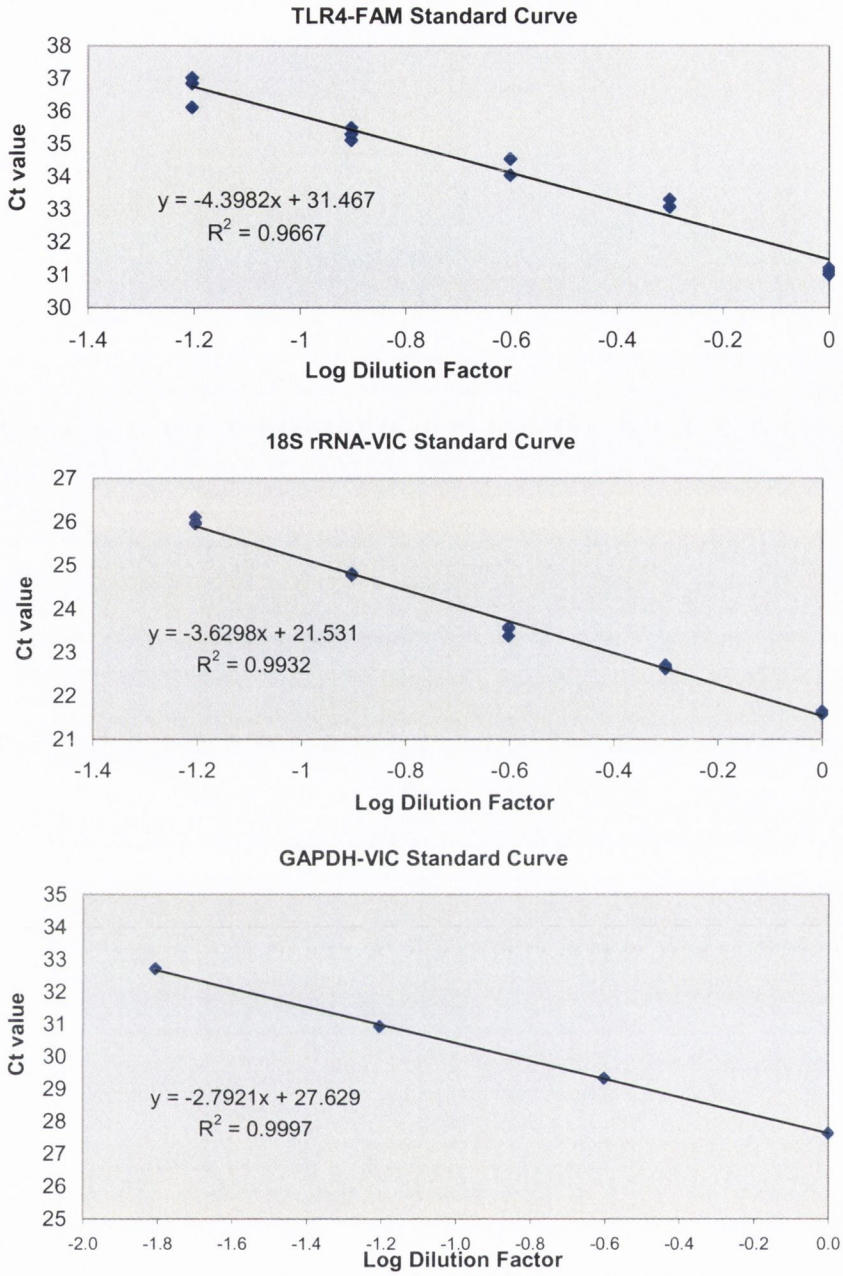

Figure 4.10 Standard curves for TLR4 (target), 18S rRNA (control), and GAPDH (control) amplification. The standard curve equation is $y = mx + b$, where m is the slope of the line and b is the y-intercept.

Relative Gene Expression of TLR4 by Real Time PCR

The relative expression of the TLR4 gene in primary human monocytes and THP1 cells treated with varying concentrations of LPS, APC, PC, and LMWH was assessed using the optimal primer and probe concentrations for real-time PCR amplification. Relative TLR4 gene expression in THP-1 cells and primary human monocytes under various conditions are shown in Figures 4.11 to 4.17. Results are averages of replicate assays and error bars indicate standard error of replicate values for each sample. The general pattern of results indicated that TLR4 gene expression is increased by LPS and/or IFN-γ treatment, and that

APC/PC or LMWH inhibits this increase. However, APC/PC or LMWH treatment alone does not significantly decrease TLR4 gene expression relative to resting levels.

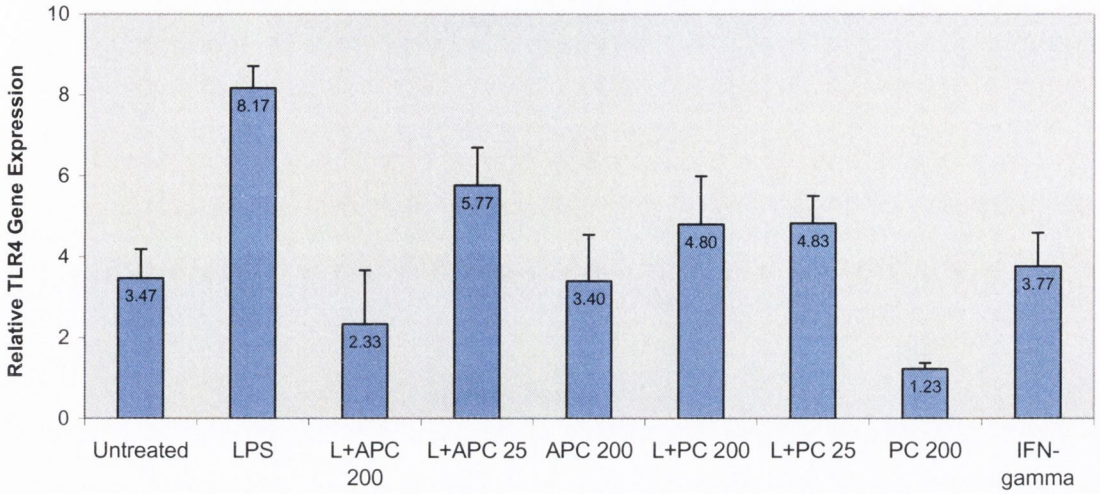

Figure 4.11 Relative TLR4 mRNA expression in primary human monocytes. Cells were pre-incubated with APC or PC (200 or 25 μg/ml) for 30 minutes, prior to LPS (10 μg/ml) or IFN-γ (500 IU/ml) addition. The experiment was stopped and RNA extracted 4 hours after LPS/IFN-γ addition. All values have been normalised against appropriate GAPDH and/or 18S RNA gene endogenous control values and results shown are the average of triplicate experiments (error bars indicate deviance from the mean value for each sample).

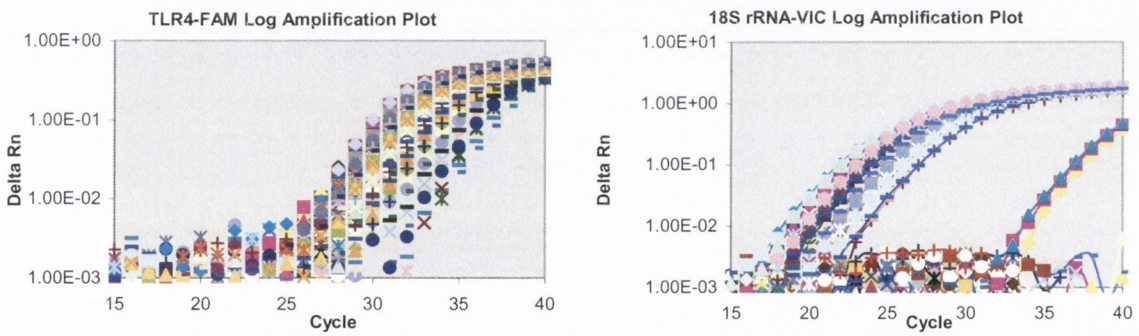

Figure 4.12 Logarithmic amplification plots of TLR4 gene (FAM) and 18S rRNA gene (VIC) real-time PCR amplification. ΔR_n is shown on the y-axis against cycle number on the x-axis.

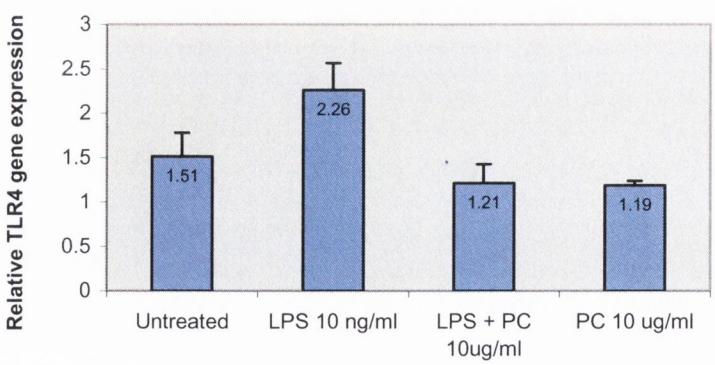

Figure 4.13 Relative TLR4 mRNA expression in THP-1 cells. Cells were pre-incubated with PC (10 μg/ml) for 30 minutes, prior to LPS (10 ng/ml) addition. The experiment was stopped and RNA extracted 90 minutes after LPS addition. All values have been normalised against appropriate GAPDH gene endogenous control values and results shown are the average of duplicate experiments (error bars indicate deviance from the mean value for each sample).

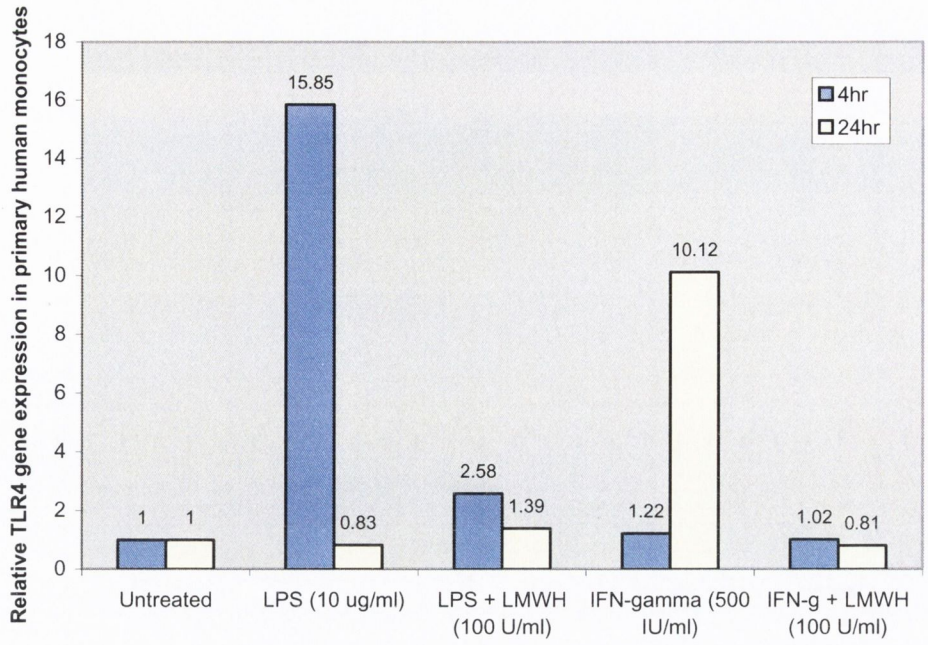

Figure 4.14 Relative TLR4 mRNA expression in LMWH, LPS, and IFN-γ treated primary human monocytes. Cells were pre-incubated with LMWH (100U/ml) for 10 minutes, prior to LPS (10 μg/ml) or IFN-γ (500 IU/ml) addition. The experiment was stopped and RNA extracted 4 hours after LPS addition. All values have been normalised against appropriate endogenous control values.

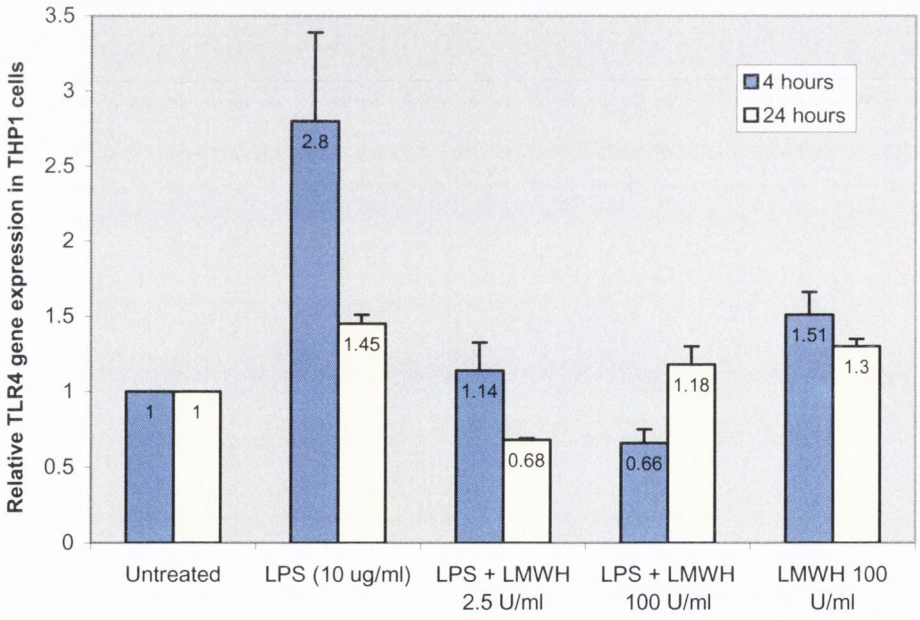

Figure 4.15 Relative TLR4 mRNA expression in LMWH and LPS treated THP-1 cells. Cells were pre-incubated with LMWH (100 or 2.5 U/ml) for 10 minutes, prior to LPS (10 μg/ml) addition. The experiment was stopped and RNA extracted 4 or 24 hours after LPS addition. All values have been normalised against appropriate endogenous control values and results shown are the average of duplicate experiments (error bars indicate deviance from the mean value for each sample).

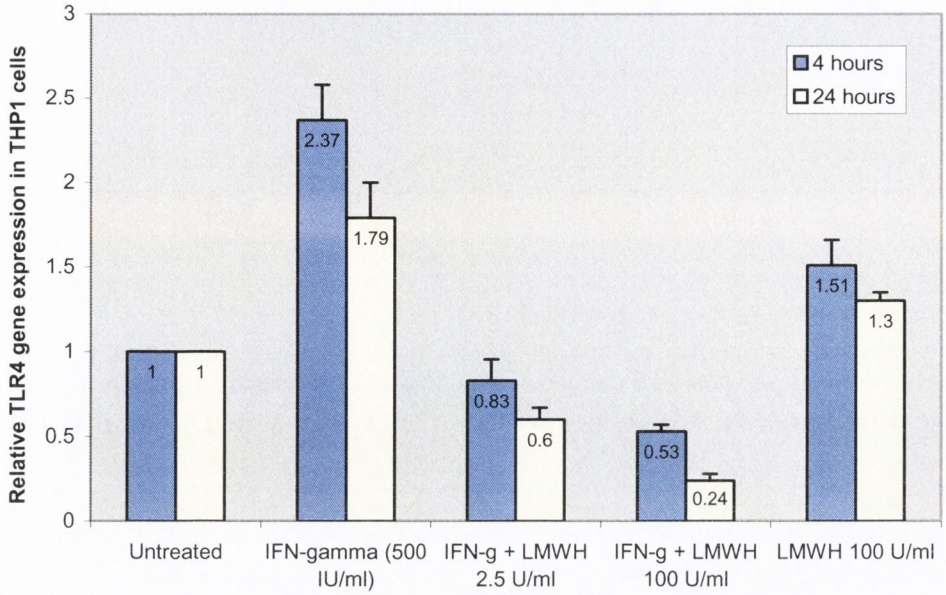

Figure 4.16 Relative TLR4 mRNA expression in LMWH and IFN-γ treated THP-1 cells. Cells were pre-incubated with LMWH (100 or 2.5 U/ml) for 10 minutes, prior to IFN-γ (500 IU/ml) addition. The experiment was stopped and RNA extracted 4 or 24 hours after IFN-γ addition. All values have been normalised against appropriate endogenous control values and results shown are the average of duplicate experiments (error bars indicate deviance from the mean value for each sample).

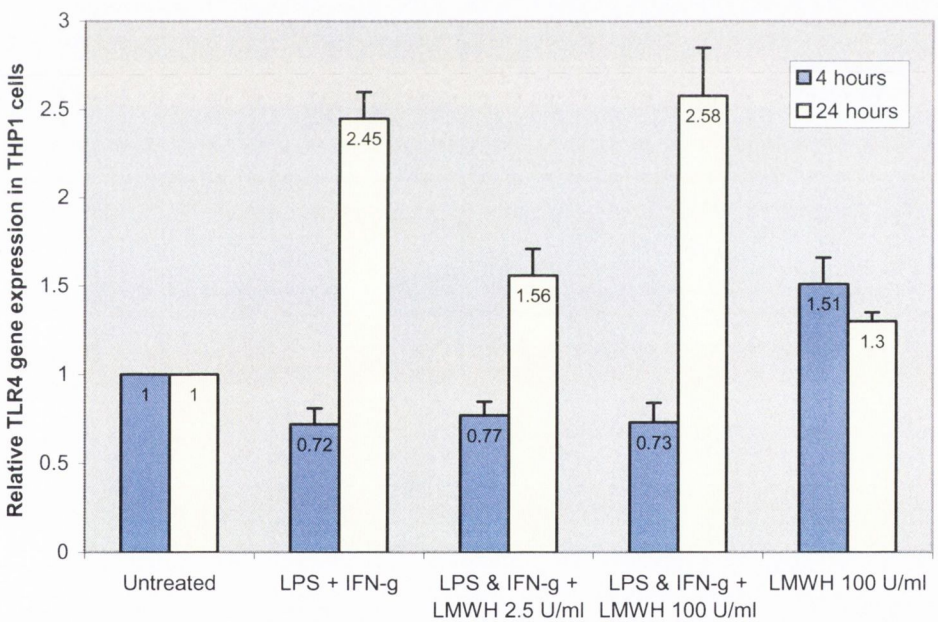

Figure 4.17 Relative TLR4 mRNA expression in LMWH and LPS + IFN-γ treated THP-1 cells. Cells were pre-incubated with LMWH (100 or 2.5 U/ml) for 10 minutes, prior to IFN-γ (500 IU/ml) and LPS (10 μg/ml) addition. The experiment was stopped and RNA extracted 4 or 24 hours after LPS/IFN-γ addition. All values have been normalised against appropriate endogenous control values and results shown are the average of duplicate experiments (error bars indicate deviance from the mean value for each sample).

4.3.3 Flow Cytometry to Investigate Regulation of TLR4 and CD14 Cell Surface Expression

Flow cytometric analysis was used to investigate possible changes in TLR4 and CD14 cell surface expression in response to a variety of stimuli. Whole blood from normal human donors was treated with various concentrations and combinations of LPS, PC, APC, or LMWH for 4 or 24 hours and associated cell surface expression of TLR4 and CD14 was assessed using a FITC-conjugated antibody to TLR4 and an RPE-conjugated antibody to CD14. The latter antibody was also utilised to gate the primary monocyte population in the whole blood samples for cell surface receptor analysis. The level of TLR4 and CD14 expression on untreated cells was used for comparison of receptor expression. As negative controls, unstained cells and cells labelled with FITC-conjugated and RPE-conjugated isotype control antibodies were also analysed. For TLR4 expression, geometric mean values of each sample were normalised by subtracting the geometric mean value of the corresponding isotype control labelled sample, thus removing effects of non-specific binding of the antibody. This was not necessary for the CD14 expression analysis as non-specific binding of this antibody was negligible (equivalent to the negative control) in comparison to the level of CD14 antibody binding. Membrane expression of TLR4 was assessed by measurement of specific FITC fluorescence in the FL1 channel whilst expression of CD14 was assessed by measurement of specific RPE fluorescence in the FL2 channel. The effect of each treatment condition (Section 4.2.3, Table 4.3) on cell surface TLR4 and CD14 levels as measured by flow cytometry is shown in Tables 4.4 to 4.11 and illustrated in Figures 4.18 to 4.22.

The Effect of APC or PC Treatment on TLR4 Cell Surface Levels

Table 4.4 Normalised geometric mean values of TLR4-FITC labelled primary human monocytes after a ½ hour incubation with APC or PC followed by LPS addition and 4 hours incubation.

Conditions	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Mean	P value	Relative % expression [c]
Untreated	6.85	7.83	8.35	8.44	8.43	7.98	0.0024 [a]	100
LPS (1 ng/ml)	9	9.54	9.2	9.51	9.85	9.42		118
LPS + PC (50 µg/ml)	7.6	7.94	7.7	10.85	9.87	8.79	0.38 [a]	110
LPS + PC (20 µg/ml)	8.36	8.26	8.75	10.76	10.57	9.34	0.89 [a]	117
LPS + APC (50 µg/ml)	7.3	6.94	7.36	10.22	10.07	8.38	0.19 [a]	105
LPS + APC (20 µg/ml)	8.38	7.74	7.86	9.27	10.03	8.66	0.14 [a]	109
PC (20 µg/ml)	6.11	7.58	7.54	8.15	8.23	7.52	0.37 [b]	94
APC (20 µg/ml)	6.18	7.93	7.24	8.56	8.67	7.66	0.56 [b]	96

[a] P value compared to LPS treated sample; [b] P value compared to untreated sample; [c] relative receptor expression on cell surface compared to untreated sample. Rep = replicate.

Table 4.5 Normalised geometric mean values of TLR4-FITC labelled primary human monocytes after a ½ hour incubation with APC or PC followed by LPS addition and 24 hours incubation.

Conditions	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Mean	P value	Relative % expression [c]
Untreated	13.47	12.92	14.89	15.79	15.22	14.46	0.0006 [a]	100
LPS (1 ng/ml)	18.6	17.81	21.07	18.35	18.29	18.82		130
LPS + PC (50 µg/ml)	13.53	13.6	15.13	13.88	15.01	14.23	0.0001 [a]	98
LPS + PC (20 µg/ml)	16.96	16.58	18.4	15.21	11	16.63	0.023 [a]	115
LPS + APC (50 µg/ml)	13.28	13.02	14.07	14.51	13.51	13.68	<0.0001 [a]	95
LPS + APC (20 µg/ml)	16.53	15.38	16.77	15.88	15.68	16.05	0.0023 [a]	111
PC (20 µg/ml)	12.84	12.83	14.08	12.86	13.26	13.17	0.062 [b]	91
APC (20 µg/ml)	12.15	13.8	12.89	13.21	13.13	13.04	0.046 [b]	90

[a] P value compared to LPS treated sample; [b] P value compared to untreated sample; [c] relative receptor expression on cell surface compared to untreated sample. Rep = replicate.

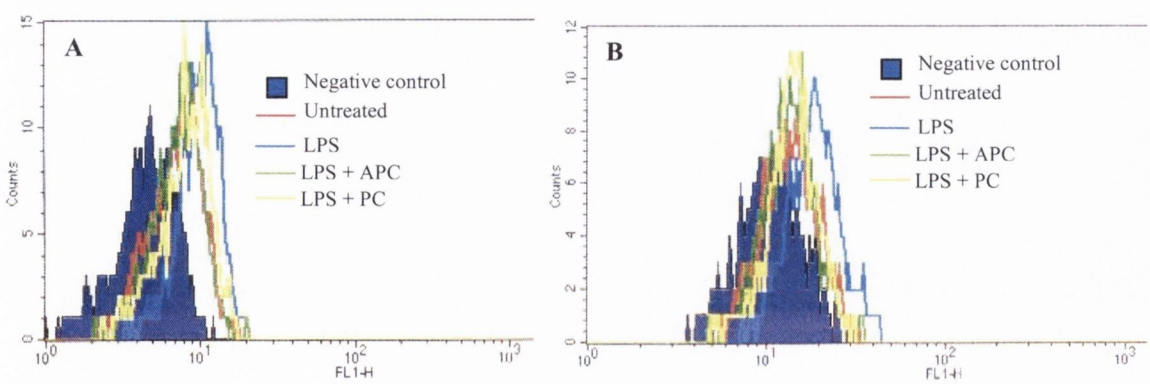

Figure 4.18 TLR4 expression on primary human monocytes in whole blood following treatment with LPS (1 ng/ml) and APC or PC (50 µg/ml) for (A) 4 hours or (B) 24 hours.

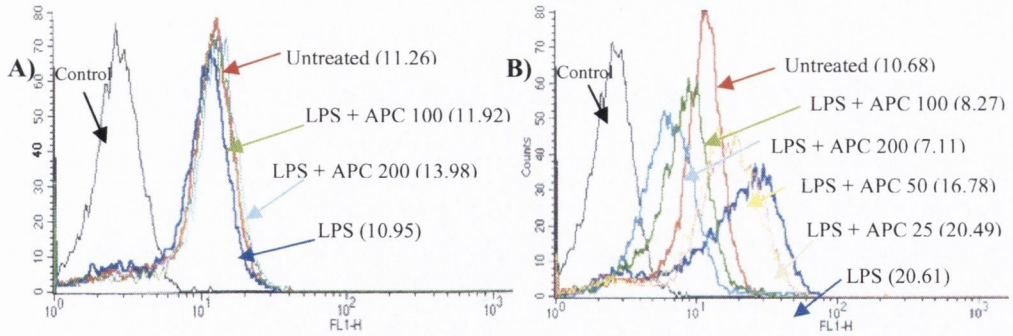

Figure 4.19 TLR4 expression on primary human isolated monocytes cultured with LPS (10 μg/ml) and APC or PC (200-25 μg/ml) for (A) 4 hours or (B) 20 hours. Mean channel fluorescence is given in brackets.

The Effect of Low Molecular Weight Heparin Treatment on TLR4 Cell Surface Levels

Table 4.6 Normalised geometric mean values of TLR4-FITC labelled primary human monocytes after a 10 minute incubation with LMWH followed by LPS addition and 4 hours incubation.

Conditions	Replicate 1	Replicate 2	Replicate 3	Mean	P value	Relative % expression [c]
Untreated	1.51	1.68	3.12	2.1	0.0044[a]	100
LPS (1 ng/ml)	6.28	8.7	6.85	7.27		346
LPS + H (1000 IU/ml)	6.48	5.76	7.35	6.53	0.44 [a]	310
LPS + H (100 IU/ml)	6.53	6.36	7.26	6.71	0.51 [a]	319
LPS + H (10 IU/ml)	6.56	7.55	8.98	7.69	0.7 [a]	366
H (100 IU/ml)	4.67	2.74	3.21	3.54	NS [b]	168

[a] P value when comparing to LPS-treated sample; [b] P value when comparing to untreated sample; [c] relative receptor expression on cell surface compared to untreated sample.

Table 4.7 Normalised geometric mean values of TLR4-FITC labelled primary human monocytes after a 10 minute incubation with LMWH followed by LPS addition and 24 hours incubation.

Conditions	Replicate 1	Replicate 2	Replicate 3	Mean	P value	Relative % expression [c]
Untreated	6.87	5.75	6	6.2	0.014[a]	100
LPS (1 ng/ml)	8.88	8.43	7.61	8.31		134
LPS + H (1000 IU/ml)	6.13	5.52	5.85	5.83	0.004 [a]	94
LPS + H (100 IU/ml)	6.89	6.04	8.68	7.2	0.23 [a]	116
LPS + H (10 IU/ml)	8.1	8.24	7.6	7.98	0.48 [a]	128
H (100 IU/ml)	4.68	4.99	4.84	4.83	0.0175 [b]	78

[a] P value when comparing to LPS-treated sample; [b] P value when comparing to untreated sample; [c] relative receptor expression on cell surface compared to untreated sample.

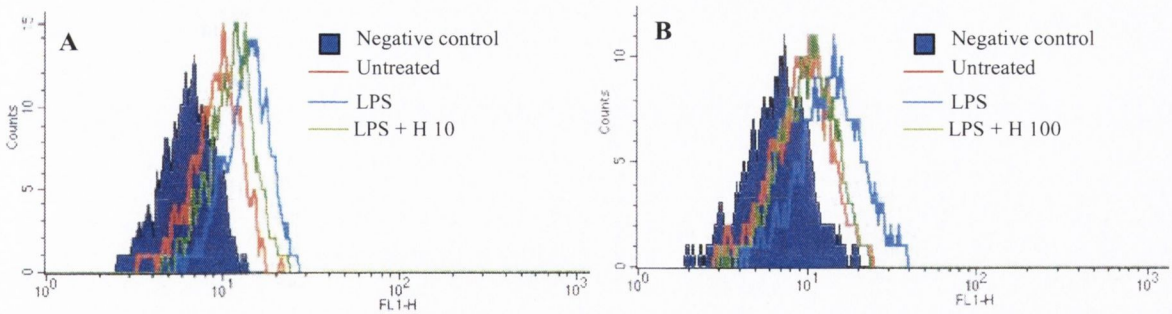

Figure 4.20 TLR4 expression on primary human monocytes following treatment with LPS (1 ng/ml) and LMWH (10 U/ml in (A) and 100 U/ml in (B)) for (A) 4 hours or (B) 24 hours.

It should be noted that the values in Tables 4.6 and 4.7 were normalised by subtracting the geometric mean value of the corresponding isotype control labelled sample in order to account for non-specific binding by the TLR4 antibody to the cell surface. Before normalisation, a greater difference was seen between LPS + LMWH treated and LPS treated samples. After four hours incubation, the average unnormalised geometric means were: untreated 13.56, LPS 19.8, LPS + LMWH (1000 U/ml) 13.83, LPS + LMWH (100 U/ml) 16.42, LPS + LMWH (10 U/ml) 19.4, and LMWH (1000 U/ml) 11.13. After 24 hours incubation, the average unnormalised geometric means were: untreated 16.62, LPS 18.31, LPS + LMWH (1000 U/ml) 13.9, LPS + LMWH (100 U/ml) 15.9, LPS + LMWH (10 U/ml) 17.9, and LMWH (1000 U/ml) 12.18. These results indicate that LMWH decreases non-specific binding by IgG2a to the cell surface. Non-specific binding is also increased to a small extent by LPS since isotype control labelled LPS treated samples generally showed an increase in mean fluorescence compared to isotype control labelled untreated samples.

The Effect of APC or PC Treatment on CD14 Cell Surface Levels

Table 4.8 Geometric mean values of CD14-RPE labelled primary human monocytes after a ½ hour incubation with APC or PC followed by LPS addition and 4 hours incubation.

Conditions	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Mean	P value
Untreated	844	429	482	347	423	1350	1470	764	0.0012 [a]
LPS (1 ng/ml)	1279	689	708	603	599	1824	1880	1083	
LPS + PC (50 µg/ml)	1387	760	699	649	626	1992	1901	1145	NS [a]
LPS + PC (20 µg/ml)	1575	650	697	529	578	1855	1808	1099	NS [a]
LPS + APC (50 µg/ml)	1428	718	686	608	779	1951	1983	1165	NS [a]
LPS + APC (20 µg/ml)	1520	678	627	580	534	1817	1912	1095	NS [a]
PC (20 µg/ml)	768	421	490	396	408	1339	1329	736	NS [b]
APC (20 µg/ml)	867	515	491	438	371	1404	1458	792	NS [b]

[a] P value when comparing to LPS-treated sample; [b] P value when comparing to untreated sample. Rep = replicate; NS = not significant.

Table 4.9 Geometric mean values of CD14-RPE labelled primary human monocytes after a ½ hour incubation with APC or PC followed by LPS addition and 24 hours incubation.

Conditions	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Mean	P value
Untreated	929	959	1011	721	751	1905	2062	1191	0.63 [a]
LPS (1 ng/ml)	1129	1275	1327	732	876	1892	2075	1329	
LPS + PC (50 µg/ml)	1146	1183	1163	1014	989	1612	1974	1297	NS [a]
LPS + PC (20 µg/ml)	1170	1256	1301	906	955	1698	1806	1299	NS [a]
LPS + APC (50 µg/ml)	1133	1257	1204	866	884	1854	1920	1303	NS [a]
LPS + APC (20 µg/ml)	1177	1269	1264	802	854	1883	1945	1313	NS [a]
PC (20 µg/ml)	958	1051	1080	767	799	1636	1912	1172	NS [b]
APC (20 µg/ml)	950	1240	1088	719	759	1876	1985	1231	NS [b]

[a] P value when comparing to LPS-treated sample; [b] P value when comparing to untreated sample. Rep = replicate; NS = not significant.

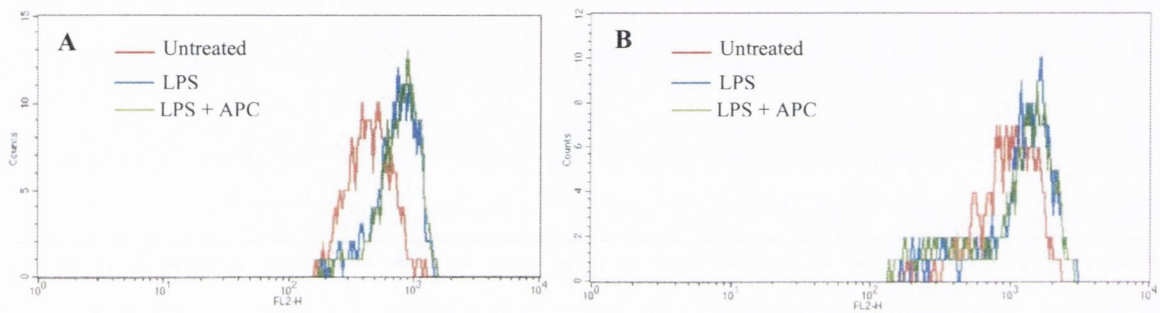

Figure 4.21 CD14 expression on primary human monocytes following treatment with LPS (1 ng/ml) and APC (50 µg/ml) for (A) 4 hours or (B) 24 hours.

The Effect of Low Molecular Weight Heparin Treatment on CD14 Cell Surface Levels

Table 4.10 Histogram geometric mean values of CD14-RPE labelled primary human monocytes after a 10 minute incubation with LMWH followed by LPS addition and 4 hours incubation.

Conditions	Replicate 1	Replicate 2	Replicate 3	Mean	P value
Untreated	1195	1009	1129	1153	0.0002 [a]
LPS (1 ng/ml)	1700	1628	1686	1699	
LPS + H (1000 IU/ml)	1593	1710	1749	1684	0.79 [a]
LPS + H (100 IU/ml)	1715	1691	1642	1683	0.71 [a]
LPS + H (10 IU/ml)	1681	1540	1672	1631	0.26 [a]
H (100 IU/ml)	1192	1097	1057	1116	0.64 [b]

[a] P value when comparing to LPS-treated sample; [b] P value when comparing to untreated sample.

Table 4.11 Geometric mean values of CD14-RPE labelled primary human monocytes after a 10 minute incubation with LMWH followed by LPS addition and 24 hours incubation.

Conditions	Replicate 1	Replicate 2	Replicate 3	Mean	P value
Untreated	1922	1909	2011	1989	0.0014 [a]
LPS (1 ng/ml)	2526	2328	2299	2418	
LPS + H (1000 IU/ml)	1819	1495	1556	1623	0.0008 [a]
LPS + H (100 IU/ml)	1633	1691	1688	1671	0.0002 [a]
LPS + H (10 IU/ml)	1956	2001	2087	2015	0.0037 [a]
H (100 IU/ml)	1124	1262	1203	1196	<0.0001 [b]

[a] P value when comparing to LPS-treated sample geometric mean values; [b] P value when comparing to untreated sample geometric mean values.

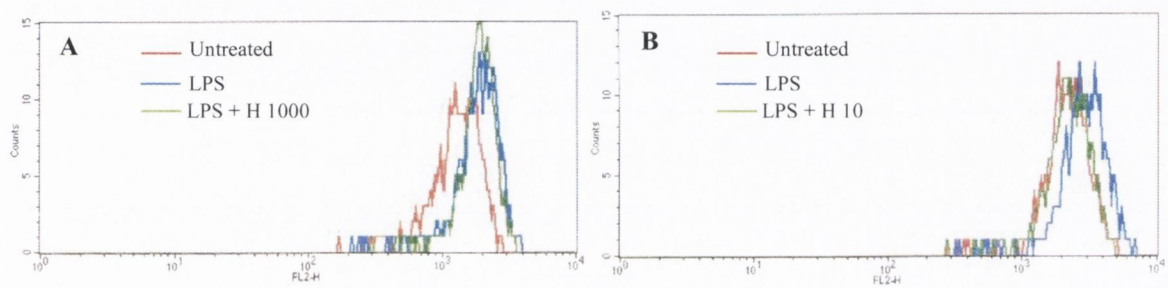

Figure 4.22 CD14 expression on primary human monocytes following treatment with LPS (1 ng/ml) and LMWH (1000 U/ml in (A) and 10 U/ml in (B)) for (A) 4 hours or (B) 24 hours.

4.4 DISCUSSION

Although protein C is now known to have anti-inflammatory effects in addition to its anticoagulant properties, the mechanism by which it exerts these effects is still to be fully elucidated. It is known that PC is capable of inhibiting the activation of monocytes by LPS, and that monocytes express TLR4, CD14, EPCR, and TM on their cell surfaces. We postulated that PC might act by binding to the monocyte cell surface *via* EPCR and causing a decrease in expression of LPS receptors TLR4 and CD14, resulting in dampening of the cell response to LPS stimulation. Due to the emergence of TLR4 as an important factor in the pathophysiology of IBD, we also postulated that LMWH might exert its positive role in the treatment of IBD by affecting TLR4 levels on macrophages recruited to the intestinal wall.

Both THP-1 cells and primary monocytes were treated with LPS, APC, PC, and LMWH, in varying concentrations and for varying times during the experiments described in this chapter and in Chapter 3. Our aim was to confirm that observed effects of such agents, for example the inhibition of cytokine production by LPS stimulated cells by APC and PC, were not simply due to the agents themselves causing cell death. We therefore determined viability and proliferation of cells treated with these agents and measured agent cytotoxicity by the methods described in Section 4.2.1. All agents allowed normal cell proliferation and were not cytotoxic with the exception of the treatment of isolated primary monocytes for 24 hours. In this case, approximately 50% or less cell viability was seen across all treatment conditions. Several factors may account for this finding. Whereas cultured cell lines are capable of growing in suspension in culture for many passages, primary monocytes can be difficult to maintain in culture due to their tendency to attach to cell culture containers, leading to difficulty in harvesting the cells without damage or stimulation, and due to their need for certain growth factors not present in the culture medium. As primary monocytes are outside of their natural environment, the blood, they are unlikely to survive for long. Additionally, the isolation procedure takes several hours and would cause some stress to the cells. Immediately after isolation the cells are stimulated and secrete inflammatory cytokines, requiring the cells to be cultured overnight to recover from stimulation before start of experiments. Thus a 24 hour experiment is concluded 48 hours after the blood is taken. However, it should be noted that for flow cytometric analysis of cell receptor levels, whole blood was treated and labelled and monocytes gated during analysis, meaning that experiments were started immediately after

blood was taken and thus the stress of the isolation procedure was avoided. Also, during the FACS analysis, cells were gated in order to exclude any dead cells and debris from the analysis.

LPS induces TLR4 surface expression on human monocytes and granulocytes, and anti-TLR4 antibodies inhibit LPS mediated TNF-α induction by approximately 70%, whilst anti-CD14 inhibit TNF-α induction nearly completely, indicating the essential nature of these receptors for cellular activation by LPS (Mirlashari and Lyberg, 2003). Additionally, TLR4 mRNA and protein levels are increased in sepsis-induced mice compared to controls, and early increases in TLR4 expression correlate with mortality whilst TLR4 gene blunting correlates with long-term survival (Williams *et al*, 2003). Since TLR4 is the primary LPS receptor and appears to be increased in sepsis and in response to LPS treatment, we wished to investigate TLR4 mRNA and protein levels in human monocytes in response to LPS and to APC and PC treatment. Real-time PCR technology was utilised to determine levels of TLR4 gene expression in human monocytes. Four hour LPS treatment increased TLR4 gene expression by approximately two-fold compared to untreated cells. This correlates with a study done by Bosisio *et al* who also found that LPS increases TLR4 mRNA expression at four and twenty four hours (Bosisio *et al*, 2002). Both APC and PC decreased LPS induced mRNA expression; however a reduction of resting TLR4 gene expression was not seen. This indicates that APC and PC are capable of reducing LPS induced gene expression as shown in chapter 3. However, PC probably does not cause this reduction of LPS induced gene expression by initially reducing levels of TLR4, an important LPS receptor and signal transducer on the monocyte cell surface. These findings are supported by the flow cytometric data which showed that a significant effect of APC or PC on either unstimulated or LPS-stimulated TLR4 receptor levels was only seen after 24 hours but was not seen after 4 hours incubation. These data indicate that reduction of TLR4 levels is unlikely to be the method by which PC reduces monocyte cell activation by LPS as shown in chapter 3, especially since effects by APC and PC on TLR4 cell surface levels were seen only after 24 hours, when decreased LPS induced gene expression by PC treatment is seen after just 90 minutes. The cell surface levels of CD14 were also investigated, as this is the initial LPS receptor which transfers LPS to TLR4 on the monocyte cell surface and a reduction in levels of this receptor would decrease the level of cell activation by LPS, dampening the cell response to LPS stimulation. However, despite an increase in CD14 levels in LPS stimulated cells, which was previously reported

(Jiang *et al*, 2000; Marchant *et al*, 1992), no effect of APC or PC on levels of this receptor was seen.

Increased levels of TLR2 and TLR4 in inflamed intestinal mucosa, localised in intestinal macrophages, has been demonstrated (Hausmann *et al*, 2002) and decreased expression of IEC TLR4 protects against dysregulated proinflammatory gene expression in response to LPS (Abreu *et al*, 2001). Since TLR4 appears to play an important role in the pathophysiology of IBD, we postulated that LMWH could work therapeutically in this disease though a reduction in the levels of this receptor on macrophages recruited to the intestinal lining or on IECs. We therefore investigated TLR4 mRNA and cell surface expression levels in unstimulated, LPS-stimulated, and IFN-γ-stimulated human monocytes and the effect of LMWH on these levels. In primary monocytes, LPS induced TLR4 gene expression after 4 hours and this induction was suppressed by LMWH. A similar pattern was seen for IFN-γ stimulation after 24 hours incubation. In THP1 cells, the general pattern of mRNA expression was similar, with a reduction of LPS- or IFN-γ-stimulated TLR4 levels by LMWH. LMWH treatment alone did not decrease unstimulated TLR4 levels. When looking at TLR4 cell surface levels, LPS treatment caused an increase both at 4 and 24 hours. LMWH treatment had no effect on this increase after 4 hours. At 24 hours, only the highest concentration of LMWH had an effect on LPS-stimulated TLR4 levels, although LMWH alone caused a significant decrease in unstimulated TLR4 levels. It was also noted that LMWH decreased non-specific binding to the monocyte cell surface. The effect of LMWH on CD14 cell surface levels was significant at 24 hours but not after 4 hours. At 24 hours, LMWH decreased LPS induced CD14 levels even at a concentration of 10 IU/ml, and resting levels were significantly higher than LMWH treated levels. From these findings, it appears that LMWH does not act primarily by reducing levels of these two LPS receptors as little effect was seen at 4 hours either on LPS induced or unstimulated cell surface levels of TLR4 and CD14. However, at high concentrations and after 24 hours incubation, a reduction of CD14, to a greater extent than that seen for TLR4 levels, does occur and may be important in the perpetuation of the therapeutic effect of LMWH in reducing the inflammatory response to LPS. It would be of interest to investigate the effect of LMWH on TLR4 levels in IECs as this may be more relevant in the pathophysiology of IBD than the effect on monocyte/macrophage TLR4 levels, as IECs are undoubtedly of great importance in this disease and the most common theory of IBD aetiology involves stimulation of the mucosal immune system by either pathogenic or lumenal bacteria, thus activating the inflammatory cascade.

An increase of TLR4 and CD14 by LPS stimulation, as seen in this study and in previous investigations, causes a prolongation of cell activation as more receptors for LPS are present on the monocyte cell surface allowing more LPS binding and signal transduction. The reduction of LPS stimulated levels of TLR4 by APC and PC and by LMWH shown here is most likely an effect of an overall reduction of LPS induced gene expression caused by these anti-inflammatory and anti-coagulant proteins. These data together indicate that APC/PC and LMWH perpetuate their anti-inflammatory effects by decreasing LPS receptor levels 24 hours after treatment. In this study it was found that APC/PC had more effect on TLR4 cell surface levels, whilst LMWH affected CD14 cell surface levels to a greater extent, perhaps indicating differing mechanisms of action by APC/PC and LMWH in achieving their anti-inflammatory effects in the monocyte.

Since it appears that APC/PC and LMWH do not function by initially reducing TLR4 or CD14 levels in order to dampen the monocyte activation by LPS, other possible actions must be considered. A potential mode of action would be interference with LPS binding to the cell surface, thus inhibiting signal transduction and induction of LPS-inducible genes. Certainly, CD11b/CD18 is part of the LPS activation cluster (Pfeiffer *et al*, 2001) and Salas *et al* demonstrated that heparin attenuates TNF-α induced inflammatory response through a CD11b dependent mechanism (Salas *et al*, 2000). Thus if heparin binds *via* CD11b, it may interfere with either formation of the LPS activation cluster on the cell surface or with LPS binding to its receptor in the activation cluster. The method by which APC/PC achieves its effects is as yet unknown but a similar mechanism to that potentially used by LMWH is likely.

CHAPTER 5

Relevance of Cytokine Gene Polymorphisms in Inflammatory and Autoimmune Diseases.

5.1 GENERAL INTRODUCTION

5.1.1 Multifactorial Disease

Many diseases that have a genetic component are caused by the additive effects of a large number of genes and environmental factors, unlike the rare cases where a disease is caused by a single gene. The expression of these multifactorial diseases can depend upon an interaction between a large number of major and minor genes at many loci throughout the genome, and can also be modulated by non-genetic factors. Genetic predisposition is poorly understood, and elucidating the genetic aetiology of a disease can be complicated by the strong influence of the environment over expression of the disease. Further difficulties in the identification of disease-associated alleles arise as these often occur almost as frequently in healthy individuals as in patients. Two approaches are commonly used to identify disease-associated genes. The first involves the targeting of candidate genes. These are usually genes that are known to play a role in processes and pathways that are involved in the disease. The second method involves the screening of multiple genetic markers across the genome, combined with linkage analysis. In this study, the former approach has been utilised.

5.1.2 Functional Polymorphisms

Polymorphisms in multiple genes may be associated with a predisposition to, or protection from, multifactorial diseases such as the autoimmune and inflammatory diseases discussed later in this chapter. Genes encoding proteins that regulate immune responses, or that are involved in the metabolism of certain compounds involved in the immune system or inflammatory response, are obvious targets for research regarding genetic susceptibility to these diseases. When investigating possible disease associated polymorphisms, those that have a functional effect are often selected for study. Functional polymorphisms are associated with changes in the level or function of a protein, usually the protein encoded by the gene in which the polymorphism is located. A polymorphism can cause a functional effect when located in an exon by causing a change in the coding sequence of the gene and

thus an amino acid substitution, or even premature truncation of the protein. A polymorphism can also have an effect when located in the regulatory sequence of a gene, such as in the promoter region. This can disrupt the binding of transcription factors, whether positive or negative regulators of gene transcription, to the promoter region, or create new binding sites for such transcription factors, leading to an up- or down-regulation of gene transcription. However, some polymorphisms that are associated with differences in protein levels or function have no clear genetic basis for this functional effect. In these cases, the genetic marker identified is possibly linked to another unidentified polymorphism causing the functional effect.

5.1.3 Cytokine Polymorphisms and Inflammatory Disease

The inflammatory response is finely balanced. The detrimental effects often associated with infectious and autoimmune diseases are thought to be caused by too intense a response. On the other hand, too weak an inflammatory reaction decreases the subsequent immune response, making the host susceptible to the effects of trauma or an invading pathogen. Key cytokines participating in the regulation of the inflammatory response include IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-6, IL-10, and TNF-α. These can be functionally divided into pro-inflammatory (IL-1, IL-6, TNF) and anti-inflammatory (IL-10, IL-1Ra) proteins. Many polymorphisms have been reported in the genes encoding these cytokines. In this study, assays were developed to genotype a set of seven such polymorphisms - six restriction fragment length polymorphisms (RFLPs) and one variable number tandem repeat (VNTR) polymorphism. Many of these polymorphisms have been associated with inflammatory and/or autoimmune diseases, some of which are listed in Table 5.1.

In this study, we postulated that small differences in cytokine expression caused by polymorphisms might have an effect on the inflammatory response to an infectious disease such as that caused by *Neisseria meningitidis*; determine disease susceptibility and phenotype of autoimmune diseases such as inflammatory bowel disease (IBD) and psoriatic arthritis (PsA); modify the systemic inflammatory response and lactic acid production after cardiopulmonary bypass; or affect the likelihood of the occurrence of stroke. Assays were developed to determine the gene frequencies of seven such polymorphisms in patients with meningococcal disease, cardiac surgery patients with and without lactic acidosis after surgery, an IBD population, a PsA population, and a stroke

Table 5.1 Cytokine gene polymorphisms: effects on gene expression and disease associations.

Gene	Polymorphism	Functional Effect	Disease Association [a]
IL-6	-174 G→C (Fishman *et al*, 1998)	• G: ↑ IL-6 in normal individuals & in reporter gene assays (LPS/IL-1 stimulation) (Fishman *et al*, 1998); in males over 60 (Bonafe *et al*, 2001); in CAD patients (Burzotta *et al*, 2001). • No association in CAD patients (Nauck *et al*, 2002) or in sepsis patients (Schluter *et al*, 2002).	S-JCA (Fishman *et al*, 1998), length hospitalisation after coronary surgery (Burzotta *et al*, 2001), CAD (Humphries *et al*, 2001), multi-infarct dementia (Pola *et al*, 2002).
TNF-β	+252 A→G (B2→B1) (TNFB) (Messer *et al*, 1991)	• B2: ↑ TNF-α expression in stimulated PBMCs (Pociot *et al*, 1991; Pociot *et al*, 1993); in severe sepsis patients (Stüber *et al*, 1996; Majetschak *et al*, 1999). • No effect on TNF-α (Messer *et al*, 1991; Whichelow *et al*, 1996; Lee *et al*, 1997). • B1: ↑ TNF-β in stimulated PBMCs from healthy controls (Messer *et al*, 1991); in stimulated PBMCs from SLE patients (Lee *et al*, 1997); in stimulated PBMCs from IDDM patients (Whichelow *et al*, 1996). • B2: ↑ TNF-β in stimulated PBMCs from healthy controls (Whichelow *et al*, 1996). • No effect on TNF-β in stimulated PBMCs (Pociot *et al*, 1993).	Outcome in severe sepsis (Stüber *et al*, 1996; Majetschak *et al*, 1999; Fang *et al*, 1999), ankylosing spondylitis (Fraile *et al*, 1998), myasthenia gravis (Zelano *et al*, 1998), IDDM (Pociot *et al*, 1993) (Whichelow *et al*, 1996).
TNF-α	-308 G→A	• A: ↑ TNF-α expression *in vitro* (Wilson *et al*, 1997; Kroeger *et al*, 1997) and *in vivo* (Warzocha *et al*, 1998; Louis *et al*, 1998; Maurer *et al*, 1999). • No effect on TNF-α response to LPS (Stüber *et al*, 1995; Fijen *et al*, 2001).	Outcome in meningococcal disease (Nadel *et al*, 1996), septic shock (Mira *et al*, 1999), cerebral malaria (McGuire *et al*, 1994), mucocutaneous leishmaniasis (Cabrera *et al*, 1995).
IL-10	-592 C→A (Turner *et al*, 1997)	• Generally no association; A: ↓ IL-10 expression in critically ill patients (Lowe *et al*, 2003).	Mortality in critically ill patients (Lowe *et al*, 2003).
IL-10	-1082 G→A (Turner *et al*, 1997)	• G: ↑ IL-10 expression in stimulated lymphocytes (Turner *et al*, 1997; Edwards-Smith *et al*, 1999); in control and CD patients (Koss *et al*, 2000); in controls (Suarez *et al*, 2003); in reporter gene studies in monocytes (Reuss *et al*, 2002). • No effect on IL-10 levels (Rees *et al*, 2002; Fijen *et al*, 2001; Ben-Ari *et al*, 2003).	IBD (Tagore *et al*, 1999), asthma severity (Lim *et al*, 1998), susceptibility to Epstein-Barr virus infection (Helminen *et al*, 1999).
IL-1RN	Intron 2 86 bp VNTR (Tarlow *et al*, 1993)	• A2: ↑ IL-1Ra & ↓ IL-1α expression in GM-CSF stimulated monocytes (Danis *et al*, 1995). • A2: ↓ IL-1Ra in cultured PBMCs (Tountas *et al*, 1999). • A2: ↑ IL-1β in cultured mononuclear cells (Santtila *et al*, 1998).	Severe sepsis (Fang *et al*, 1999), Multiple sclerosis (Crusius *et al*, 1995), IBD (Mansfield *et al*, 1994), SLE (Blakemore *et al*, 1994).
IL-1β	+3953 C→T (Pociot *et al*, 1992)	• T: ↑ IL-1β expression in LPS-stimulated monocytes (Pociot *et al*, 1992). • No effect on *in vivo* IL-1β levels (Dominici *et al*, 2002; Mark *et al*, 2000).	Early-onset periodontal diseases (Parkhill *et al*, 2000), IBD (Nemetz *et al*, 1999), tuberculosis (Wilkinson *et al*, 1999), alopecia areata (Galbraith *et al*, 1999).

[a] Not all published disease associations are included in this table.

↑ or ↓ = increase or decrease in expression compared to other alleles of the same polymorphism; → = single base/amino acid substitution; CAD = coronary artery disease; S-JCA = Systemic-onset juvenile chronic arthritis; IDDM = insulin dependent diabetes mellitus; IBD = inflammatory bowel disease; SLE = systemic lupus erythematosus; CD = Crohn's disease.

population. In addition, a large control population was genotyped. The aim of this study was to determine if any of these polymorphisms is associated with outcome or severity in the diseases mentioned above. Cytokine levels were also measured in a group of normal controls in order to confirm or elucidate functional effects of these polymorphisms.

5.2 GENERAL MATERIALS AND METHODS

5.2.1 Subjects

All patients and controls included in this study were Caucasian. Study protocols are dealt with in detail in each section.

Controls

The control population for genotyping (Sections 5.4 – 5.8) consisted of 389 Irish blood donors (male 58%, female 42%, mean age 37.1, range 18-65 years) collected from The Northern Ireland Blood Transfusion Service (n=60) and The Blood Transfusion Service Board (Dublin, Cork, and mobile units throughout the Republic of Ireland) (n=329).

5.2.2 DNA Isolation

Control Samples

DNA was isolated from the EDTA-anticoagulated donor bloods using a rapid large volume extraction protocol. Briefly, whole blood was washed 3 times in 10mM NaCl, 10mM EDTA (pH 7.0), pelleted, and resuspended in 1.25 ml of 200mM NaOH. Following incubation at 97^{o}C for 15 minutes, the sample was neutralised with an equivalent volume of 200mM HCl, 100mM Tris-HCl (pH 8.5).

Invasive Meningococcal Disease Samples

DNA was isolated from the meningococcal sepsis blood samples using the Puregene extraction procedure (Gentra Systems, Minneapolis, USA) according to the manufacturer's instructions.

Cardiopulmonary Bypass Samples, IBD Samples, Psoriatic Arthritis Samples, Stroke Samples, Control Samples (Cytokine Plasma Level Study Section 2.3)

Whole blood was collected in tubes containing ethylenediaminetetraacetic acid (EDTA) for the study groups. Control blood samples were collected in tubes containing citrate (10%). Genomic DNA was extracted from the blood samples by a standard phenol/chloroform method (Section 2.2.4).

5.2.3 Polymerase Chain Reaction

Amplification of polymorphic sites of interest was carried out as detailed in Section 2.2.4. All primers were synthesised commercially (Sigma-Genosys) (Table 5.2). The cycling parameters for each assay are listed in Table 5.3, along with any changes to the standard PCR reaction mix. Details of primer sites and cut sites in relevant genes, along with examples of RFLP digestion products run in agarose gel for each RFLP assay, are shown in Appendix A.

5.2.4 Restriction Enzyme Digestion

All restriction enzymes, listed in Table 5.2, were obtained from New England BioLabs (Beverly, MA, USA), except *Hsp 92 II* which was purchased from Promega. Each enzyme was supplied with the appropriate restriction enzyme buffer, and BSA where necessary. Digestions were carried out at the temperature recommended by the manufacturer. The amplified IL-6 product (244 bp) was digested in a reaction mix containing 22 µl PCR product, 2 units of *Hsp92 II*, 100 µg/ml BSA, and restriction Buffer K (10 mM Tris-HCl (pH 7.4), 0.15 M KCl, 10 mM $MgCl_2$) overnight at 37°C. The amplified area of the TNF-α promoter containing the TNF-α -308 polymorphism (195 bp) was digested in a 20 µl total reaction mix containing 18 µl PCR product, 2.5 units of *Nco I*, and NE Buffer 4 (50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate, 1 mM DTT) for 6 hours or overnight at 37°C. Amplicons of the area of the TNF-β gene containing the TNFB polymorphism (782 bp) were digested in a 20 µl reaction volume containing 18 µl PCR product, 5 units of *Hinf I*, and NE Buffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM Mg Cl_2, 1 mM DTT) for 3 hours at 37°C. The PCR product containing the IL-1β polymorphism (258 bp) was digested in a 20 µl volume containing 17.3 µl PCR product, 10 units of *Taq*$^{\alpha}$ *I*. 100 µg/ml BSA, NE Buffer Taq$^{\alpha}$ I (100 mM NaCl, 10 mM Tris-HCl, 10 mM Mg Cl_2) for 12 hours at 65°C. The amplified IL-10 -1082 product (190 bp) was digested in a total volume of 25 µl containing 22 µl PCR product, 1 unit *Mnl I*, 100 µg/ml BSA, and NE Buffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM Mg Cl_2, 1 mM DTT) for 6 hours or overnight at 37°C. The area of the IL-10 promoter region containing the IL-10 -592 polymorphism (437 bp) was digested in a 20 µl total volume containing 18 µl PCR product, 2 units of *Rsa I*, and NE Buffer 1 (10 mM Bis Tris Propane-HCl, 10 mM Mg Cl_2, 1 mM DTT) for 6 hours or overnight at 37°C.

Table 5.2 PCR primer sequences with position of 5' base, restriction enzymes, and cut sites. Primers designed for the purposes of this study unless otherwise noted.

Primer	Sequence (5'-3')	Position of 5' Base	Restriction Enzyme	Restriction Sites	
				Variant	Constant
IL-6 –174 sense	ATGACTTCAGCTTTACTCTT	-324	*Hsp 92 II*	-174	-296
IL-6 –174 antisense	ATAAATCTTTGTTGGAGGGT	-81			
TNFB sense [a]	CCCTCCTGCACCTGCTGCCTGG	+112	*Hinf I*	+251	+196
TNFB antisense [a]	AGAGGGG TGGATGCTTGGGTTC	+833			
TNF-α –308 sense	GGAGGCAATAGGTTTTGAGGGCCAT	-334	*Nco I*	-313	-162
TNF-α -308 antisense	CTGTCTCGGT TTCTTCTCCATGGCG	-140			
IL-10 –1082 sense	TCTGAAGAAGTCCTGATGTC	-1248	*Mnl I*	-1085	- 1196, -1191
IL-10 –1082 antisense	CTCTTACCTATCCCTACTTCC	-1059		-1081	-1174
IL-10 –592 sense	GACTCCAGCCACAGAAGCTTA	-967	*Rsa I*	-594	-884, -842,
IL-10 –592 antisense	ATATCCTCAAAGTTCCCAAGC	-531			-834
IL-1β +3953 sense	GTGTTGTCATCAGACTTTGACCGTA	+3816	*Taq I*	+3952	+4052
IL-1β +3953 antisense	GAGAGCTTTCAGTTCATATCGACCA	+4073			
IL-1RN sense [b]	CTCAGCAACACTCCTAT	Intron 2	N/A	N/A	N/A
IL-1RN antisense	GCAGCAATAATGAAGAG	Intron 2			

[a] TNFB primers from (Stüber *et al*, 1996).
[b] IL-1RN sense primer from (Fang *et al*, 1999).

Table 5.3 Polymerase chain reaction cycling parameters, changes to standard reaction mix, and percentage agarose gel used in each assay. Cycling parameters optimised in this study unless otherwise noted.

Polymorphism	Cycles	Denaturation	Annealing	Elongation	Changes to Standard Reaction Mix	Agarose Gel
IL-6 –174	40	94°C, 1min	58°C, 1min	72°C, 90 sec		3%
TNFB [a]	37	95°C, 30 sec	68°C, 30 sec	74°C, 42 sec	1μM each primer	2%
TNF-α –308	40	94°C, 1 min	65°C, 1 min	72°C, 1 min		2%
IL-10 –1082	40	94°C, 1 min	58°C, 1 min	72°C, 1 min	No DMSO	4%
IL-10 –592	40	94°C, 1 min	64°C, 1 min	72°C, 1 min	1μM each primer	3%
IL-1β +3953	40	94°C, 1 min	65°C, 1 min	72°C, 1 min		3%
IL-1RN 86bp VNTR	40	94°C, 1 min	58°C, 1 min	72°C, 90 sec	1μM each primer; 6% DMSO	2%

[a] TNFB cycling parameters from (Stüber *et al*, 1996).

5.2.5 Agarose Gel Electrophoresis

Restriction digest products were run in the appropriate percentage of agarose gel (Table 5.3) as detailed in Section 2.2.4.

5.2.6 Statistical Analysis

Allele and genotype frequencies were compared between categorical variables (e.g., patient and control groups, and patient sub-populations) by means of the chi-squared (χ^2) test and Fischer's exact test, when appropriate. Odds ratios and the corresponding 95% CI were estimated by cross-tabulation. Association between continuous variables (e.g. age of disease onset or lactic acid levels) and categorical variables (e.g. genotype) was determined by a one-way analysis of variance (ANOVA) test and Student's t test. Where association between an individual polymorphism and lactic acid levels was detected on such a univariate test, then the interaction between polymorphism and change in lactic acid levels with time was analysed by multivariate ANOVA (MANOVA). Where data was not normally distributed, such as in the study of serum cytokine levels, continuous variables, (e.g. cytokine level) were compared to categorical variables (e.g. genotype) by means of the non-parametric Mann-Whitney test (when comparing just two categorical variables) or by means of the Kruskal-Wallis test (when comparing more than two categorical variables). Nominal logistic regression analysis was performed to assess the influence of multiple polymorphisms on disease outcome. Genotype distributions were tested using the Hardy-Weinberg equilibrium. All statistics were performed using JMP software (JMP version 4.0.4, SAS Institute Inc, Cary, NC, USA) or Statview software (Statview version 4.5, Abacus Concepts Inc, Berkeley, CA, USA).

P values of <0.05 were considered statistically significant. The p values were not adjusted for multiple comparisons as it was not considered appropriate to apply this conservative adjustment to an exploratory candidate gene study such as this, where careful interpretation of any p-values is necessary and where sufficient detail is provided for the reader to make his/her own adjustment. It was therefore thought most appropriate to provide unadjusted p-values and to provide sufficient detail for the reader to make his/her own adjustment, rather than to increase the complexity of the results shown. Also, individual alleles investigated in this study were not independent of each other, and previous studies revealed some of the genes investigated here to be associated with the occurrence of the disease (see Table 5.1,

Chapter 5). A Bonferroni correction would therefore be a very conservative criterion to apply to the results. Other similar studies to this one do not adjust for multiple comparisons (Middleton *et al*, 1998; van der Pol *et al*, 2001; Wu *et al*, 2002).

5.3 ASSOCIATION BETWEEN CYTOKINE PROTEIN LEVELS AND CYTOKINE POLYMORPHISM GENOTYPE

5.3.1 Introduction

Most of the polymorphisms investigated in this study have previously been reported to be associated with an increase or decrease in the level of protein production by the gene in which they occur, although some of the reports have been conflicting. Each of the seven polymorphisms are dealt with in detail below.

IL-6 G-174C

IL-6 gene expression is tightly controlled, and the promoter region contains many transcription regulatory sites. A single base pair G to C transition at position –174 in the 5' flanking region of the IL-6 gene is located in an area of the promoter thought to have a negative effect on gene transcription (Fishman *et al*, 1998). It has been reported that the C allele of this polymorphism results in lower IL-6 expression after inflammatory stimulus compared to the G allele, and the C allele has also been found to be associated with lower resting plasma IL-6 levels in normal individuals (Fishman *et al*, 1998). Reports on the functionality of this polymorphism since the study by Fishman *et al* have been varied: Nauck *et al* found no difference in plasma IL-6 concentration between groups with different IL-6 genotypes in 942 individuals with coronary artery disease (Nauck *et al*, 2002); Bonafe *et al* looked at serum levels in 700 individuals aged 60 to 110 years of age and found an association between levels and genotype only in males (Bonafe *et al*, 2001); Kilpinen *et al* found an association between genotype and IL-6 plasma levels in neonates, however the CC genotype was associated with increased IL-6 in this study (Kilpinen *et al*, 2001); Burzotta *et al* looked at 111 patients with coronary artery disease and found an association between the GG genotype and higher plasma IL-6 (Burzotta *et al*, 2001); and Schluter *et al* found no association between serum IL-6 levels and genotype in sepsis patients (Schluter *et al*, 2002).

TNF-α G-308A

The TNF-α gene contains a G to A transition at position –308 in the gene promoter region. TNF2 (A), the less common allele of this polymorphism, is associated with higher constitutive and inducible levels of TNF-α secretion both *in vitro* (Wilson *et al*, 1997; Kroeger *et al*, 1997) and *in vivo* (Warzocha *et al*, 1998; Louis *et al*, 1998; Maurer *et al*,

1999). However, it has also been reported that the polymorphism has no functional effect on the response of the TNF gene to bacterial endotoxin (Stüber *et al*, 1995; Fijen *et al*, 2001).

TNF-β G+252A

The biallelic polymorphism TNFB, caused by a G to A transition at position +252 in intron 1 of the TNF-β gene, is linked to an amino acid variation at position 26 (exon 2) in the TNF-β sequence, where asparagine is associated with the B1 allele and threonine with the B2 allele (Messer *et al*, 1991). The functional effects of this polymorphism seem to vary depending on the population studied. It has been found that LPS- and phytohemagglutinin (PHA)-induced human monocytes and PBMCs from TNFB2 (A allele) homozygous individuals have a greater TNF-α secretory capacity than monocytes or PBMCs from either heterozygotes or TNFB1 homozygous individuals (Pociot *et al*, 1991) (Pociot *et al*, 1993), and severe sepsis patients that are TNFB2 homozygous have been found to have higher circulating levels of TNF-α (Stüber *et al*, 1996; Majetschak *et al*, 1999). However, no correlation between TNFB genotype and TNF-α levels has been reported (Messer *et al*, 1991; Whichelow *et al*, 1996; Lee *et al*, 1997). The effect of the TNFB polymorphism on TNF-β levels is also unclear. In PHA stimulated PBMCs, the B1 allele has been associated with higher levels of TNF-β (Messer *et al*, 1991; Lee *et al*, 1997). In contrast, the opposite trend has been reported in healthy controls where the B2B2 genotype was associated with increased TNF-β production, although, in the same study, in IDDM patients, the B1B1 genotype was found to be associated with higher TNF-β levels (Whichelow *et al*, 1996). Additionally, no correlation between TNFB genotype and TNF-β production has been reported (Pociot *et al*, 1993).

IL-10 G-1082A and IL-10 C-592A

Two single base pair polymorphisms in the promoter region of the IL-10 gene at positions -1082 and -592 relative to the transcriptional start site lie within an ETS-like recognition site. The IL-10 -1082 G allele has been associated with higher IL-10 levels compared to the A allele in Con A stimulated cultured human lymphocytes (Turner *et al*, 1997; Edwards-Smith *et al*, 1999) in plasma of controls and Crohns disease (CD) patients (Koss *et al*, 2000), and at the mRNA and serum protein level in normal controls (Suarez *et al*, 2003). In human monocytes, the IL-10 -1082 A allele was found to show higher binding affinity to the transcription factor PU.1 resulting in decreased transcriptional activity of –

1082 A haplotypes (Reuss *et al*, 2002). However, a study by Rees *et al*, found that -1082 A allele confers a twofold increase in transcriptional activity of the IL-10 promoter compared to the G allele in an Epstein-Barr transformed B cell line, and the same study found no consistent effect of genotype on IL-10 production by PBMCs (Rees *et al*, 2002). A few others studies have also reported no association between IL-10 -1082 genotype and IL-10 levels (Fijen *et al*, 2001; Ben-Ari *et al*, 2003). The IL-10 -592 polymorphism has not been found to have a functional effect on IL-10 levels in many studies, although a link between the –592 A allele and lower IL-10 levels was found in one study, which also found no association between IL-10 –1082 genotype and IL-10 levels (Lowe *et al*, 2003). This polymorphism at -592, along with another at position -819, is in linkage disequilibrium with the –1082 polymorphism, due to their close proximity in the IL-10 promoter region, and three haplotypes have been described that are formed by the -1082, -819, and -592 SNPs: GCC, ACC, ATA (Turner *et al*, 1997). The majority of studies have found the GCC haplotype to be associated with higher IL-10 levels compared to the other two haplotypes (Turner *et al*, 1997; Suarez *et al*, 2003; Edwards-Smith *et al*, 1999; Hulkkonen *et al*, 2001), explaining why -592 genotype has not been linked to higher or lower IL-10 levels in most studies.

IL-1β C+3953T

A bi-allelic polymorphism at position +3953 in exon 5 of the IL-1β gene, a Taq I RFLP, was initially reported to influence the level of stimulated IL-1β production (Pociot *et al*, 1992), the rarer T allele being associated with higher IL-1β levels in LPS- and PHA-stimulated human monocytes, but not in non-stimulated monocytes. The T allele was also associated with increased production of IL-1β by activated PBMCs from periodontitis patients, although this association was not statistically significant (Gore *et al*, 1998). However, two other studies reported that this polymorphism was not associated with *in vitro* protein levels of IL-1β (Dominici *et al*, 2002; Mark *et al*, 2000).

IL-1RN 86bp VNTR

A penta-allelic polymorphism in intron 2 of the IL-1RN gene (encoding IL-1Ra) is caused by various numbers of an 86 base pair tandem repeat (VNTR) (Tarlow *et al*, 1993). Allele 2, which has the least number of tandem repeats, has been shown to be associated with enhanced production of IL-1Ra and reduced production of IL-1α by GM-CSF stimulated human monocytes (Danis *et al*, 1995), but has also been reported to be associated with decreased production of IL-1Ra protein in cultured PBMCs (Tountas *et al*, 1999). Hurme

et al found an increase in mean plasma IL-1Ra levels in carriers of the A2 allele, however this association required the absence of the IL-1β +3953 T allele or the presence of the IL-1β –511 allele 2 (Hurme and Santtila, 1998), and Santtila *et al* found the A2 allele of the IL-1RN polymorphism to be associated with increased *in vitro* IL-1β levels (Santtila *et al*, 1998).

In order to confirm previous reported results, and to elucidate the functionality of the polymorphisms whose effects have not been reported or where published studies shown conflicting results, levels of IL-6, TNF-α, IL-1β, and IL-10 protein were measured in the serum of a group of normal controls, who were also genotyped for the seven cytokine polymorphisms. Mean cytokine levels were then compared to genotype in order to determine if there was a difference in mean plasma cytokine levels between individuals with different genotypes.

5.3.2 Subjects and Methods

Subjects

The control population used for genotyping and measuring blood cytokine protein levels consisted of 65 healthy volunteers collected from amongst staff at the Rotunda Hospital, Dublin, and the Department of Genetics, Trinity College Dublin.

Measurement of Serum Cytokine Levels

Blood samples (6 ml) were collected from 65 healthy volunteers in serum clotting activator separation gel tubes (Sarstedt, Wexford, Ireland), spun for 10 minutes at 2000 rpm, aliquoted into 300 µl samples and stored at –70ºC until used for ELISA. A separate sample of blood, approximately 3 ml, was collected in 10% citrate tubes (Sarstedt, Wexford, Ireland), and used for DNA extraction (Section 2.2.4) and PCR/RFLP analysis.

5.3.3 Results

Tumour Necrosis Factor-α

The distribution of TNF-α serum levels across the control group varied, with some individuals having highly elevated levels compared to the bulk of the group (Figure 5.1a). Serum TNF-α levels were compared with both the TNF-α -308 and TNFB genotypes. The frequency of the TNF-α -308 A allele was low, resulting in few AA individuals (n=8;

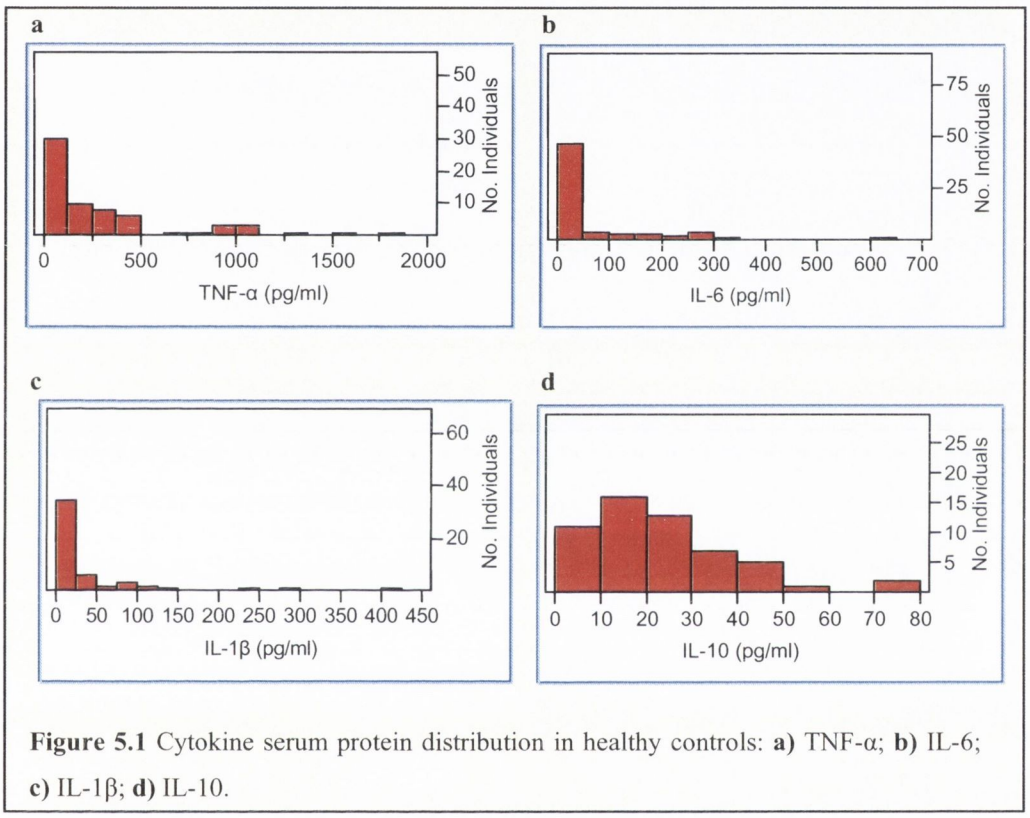

Figure 5.1 Cytokine serum protein distribution in healthy controls: **a)** TNF-α; **b)** IL-6; **c)** IL-1β; **d)** IL-10.

12%). Due to the low frequency of AA homozygotes, the most common method used in previous studies has involved studying carriage of the A allele, rather than comparing all three genotypes (Louis *et al*, 1998; Mira *et al*, 1999). Therefore, in this study, individuals with the AA genotype were grouped with heterozygotes (GA), in order to compare the mean serum TNF-α level to that of GG individuals. A difference in mean TNF-α levels was observed between these two groups, with the GA+AA group having a higher mean TNF-α level (378 ± SE 82 pg/ml) than the GG group (239 ± SE 63 pg/ml) (Figure 5.2 a). However, this difference in mean TNF-α serum levels did not reach statistical significance (p=0.35, Mann-Whitney). A second approach for the analysis of this data was to group individuals on the basis of low TNF-α levels (below the total group mean of 305 pg/ml) or high TNF-α levels (above the group mean). 24% of GG individuals were found to have TNF-α levels above 305 pg/ml, whereas 52% of GA or AA individuals were in this group (χ^2 p value=0.019).

For the TNFB polymorphism, B1B1 individuals (n=13, 20%) were grouped with B1B2 individuals. This B1B1+B1B2 group was found to have a higher mean TNF-α serum level (363 ± SE 76 pg/ml) than the B2B2 group (206 ± SE 48 pg/ml), although this difference was not statistically significant (p=0.8, Mann-Whitney) (Figure 5.2 b). When grouping individuals on the basis of TNF-α serum levels above or below the group mean (310 pg/ml), 39% of B1B1 or B1B2 individuals had TNF-α serum above this level, whereas 29% of B2B2 individuals were in this high TNF-α producing group (χ^2 p value=0.42).

Interleukin-6

IL-6 serum levels were not normally distributed, with a high proportion of individuals with IL-6 levels between 0 and 50 pg/ml (Figure 5.1 b). Due to the small size of the CC group (n=7, 11%), all GC and CC individuals were grouped together. The GG group was found to have a higher mean IL-6 protein level (95 ± SE 28 pg/ml) than the GC+CC group (34 ± SE 10 pg/ml) (p=0.09, Mann-Whitney) (Figure 5.2 c). The total mean IL-6 serum level was 60 pg/ml. It was found that 39% of individuals with the GG genotype had IL-6 serum levels above the group mean, compared to the group of individuals with the GC or CC genotype, 19% of which had IL-6 serum levels above 60 pg/ml (χ^2 p value=0.07).

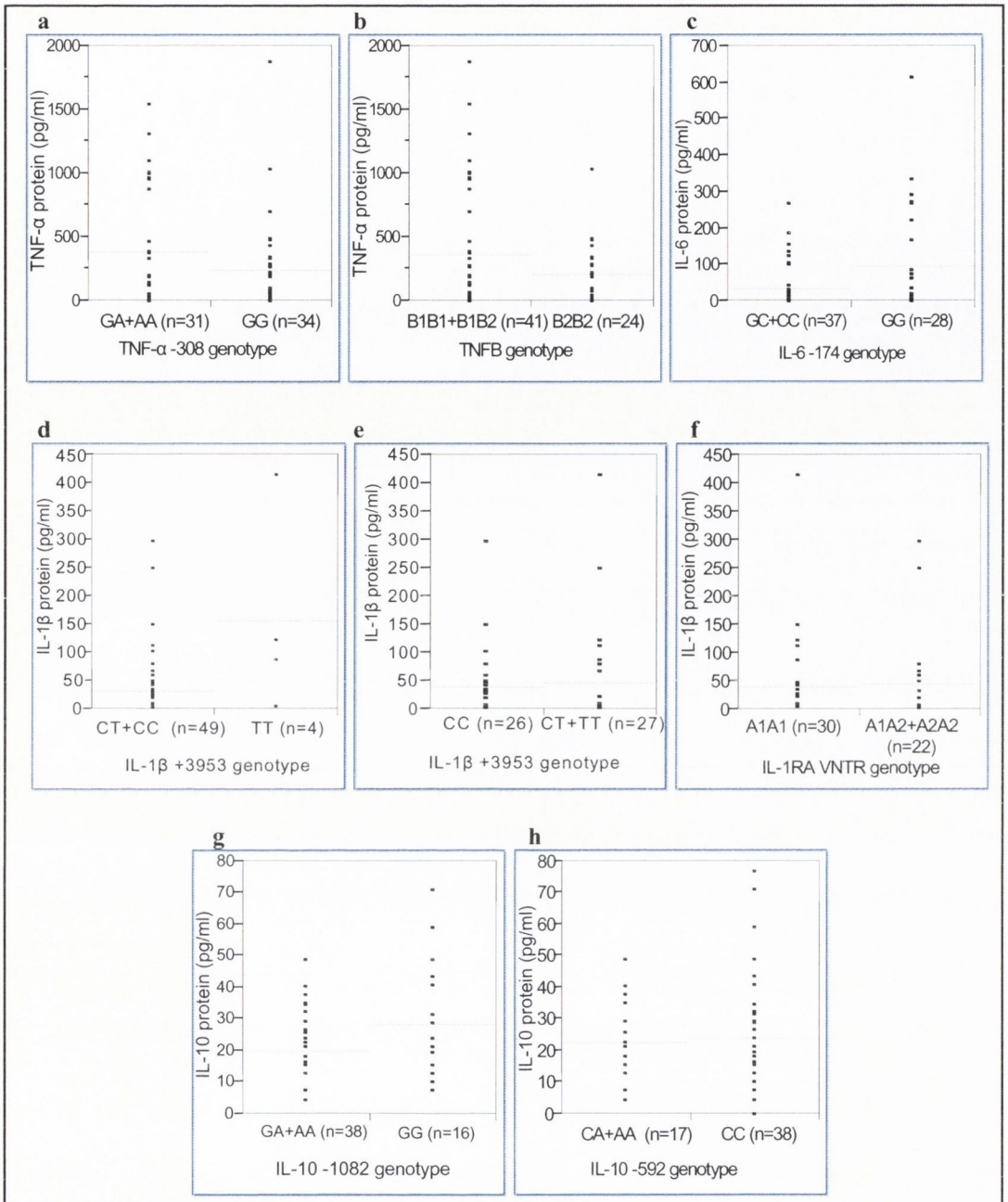

Figure 5.2 Mean cytokine serum protein levels compared to genotype in healthy controls (mean lines are shown in green): **a)** TNF-α levels compared to TNF-α -308 genotype (p=0.35, Mann-Whitney); **b)** TNF-α levels compared to TNFB genotype (p=0.8, Mann-Whitney); **c)** IL-6 levels compared to IL-6 -174 genotype (p=0.09, Mann-Whitney); **d & e)** IL-1β levels compared to IL-1β +3953 genotype (p=0.026 & p=0.25 respectively, Mann-Whitney); **f)** IL-1β levels compared to IL-1RA VNTR genotype (p=0.8, Mann-Whitney); **g)** IL-10 levels compared to IL-10 -1082 genotype (p=0.19, Mann-Whitney); **h)** IL-10 levels compared to IL-10 -592 genotype (p=0.84, Mann-Whitney).

Interleukin-1β

IL-1β protein serum levels were not normally distributed across the control group, as was the case for TNF-α and IL-6 levels (Figure 5.1 c). Individuals with the TT genotype had higher mean IL-1β protein levels (156 ± SE 89 pg/ml) compared to both other genotypes grouped together (32 ± SE 9 pg/ml) (p=0.026, Mann-Whitney) (Figure 5.2 d). When grouping the CT and TT groups together, due to the small size of the TT group (n=4, 7.5%), the mean was found to be slightly higher in this group (45.17 ± SE 17.87 pg/ml) than in the CC group (37.83 ± SE 12.48 pg/ml), although not significantly different (p=0.25, Mann Whitney) (Figure 5.2 e). Using the alternative method of looking at IL-1β levels in comparison to the whole group mean (42 pg/ml), 75% of TT individuals were above this level, compared to 24% of CT or CC individuals (χ^2 p value=0.03). However, grouping the TT and CT individuals together, 26% were above the mean IL-1β level, compared to 31% of CC individuals (χ^2 p value=0.7).

When investigating the IL-1RN 86 bp VNTR, one individual was found to have the A1A3 genotype, and was excluded from the study. Since only four individuals had the A2A2 genotype, these individuals were grouped with the A1A2 individuals. The A1A1 group had a slightly lower mean IL-1β level (38 ± SE 15 pg/ml) than the A1A2+A2A2 group (44 ± SE 17 pg/ml), but this was not a statistically significant difference (p=0.82, Mann Whitney) (Figure 5.2 f). In the A1A1 group, 23% had mean IL-1β levels above the total group mean (42 pg/ml), while 32% of the A1A2 or A2A2 group had mean levels above this amount (χ^2 p value=0.5).

Interleukin-10

The distribution of IL-10 protein levels across the group is shown in Figure 5.1 d. IL-10 levels were compared with both IL-10 –1082 and IL-10 –592 genotypes. For the –1082 polymorphism, GG individuals had the highest mean IL-10 levels (28 ± SE 5 pg/ml), GA individuals had the lowest (18 ± SE 2 pg/ml), and AA individuals (the smallest group, n=11, 20%) had an intermediate mean level (25 ± SE 4 pg/ml). Grouping GA and AA individuals, the mean IL-10 level (20 ± SE 2 pg/ml) was lower than the GG group mean level (p=0.19, Mann Whitney) (Figure 5.2 g). This GA+AA group was found to have 42% of individuals with an IL-10 level above the mean IL-10 level (22 pg/ml) compared to 50% of individuals in the GG group (χ^2 p value=0.37).

The study group included two individuals with the IL-10 –592 AA genotype (4%) and these were grouped with the CA individuals. This CA+AA group had a similar mean IL-10 level (22.6 ± SE 3 pg/ml) to the CC group (23.4 ± SE 3 pg/ml) (p=0.84, Mann Whitney) (Figure 5.2 h), and there were 47% and 42% of individuals with mean IL-10 levels above the group mean (23.2 pg/ml) in the two groups respectively (χ^2 p value=0.95).

Summary of Results

All serum cytokine protein level results are shown in Table 5.4, including means and standard errors, medians, percentage of individuals above the total group mean, and percentage of individuals above the total group median, for each genotype and grouped genotypes of the seven polymorphisms under investigation.

5.3.4 Discussion

This study was undertaken in order to determine if there is an association between seven cytokine polymorphisms and levels of serum cytokine proteins, as has previously been reported. Despite finding considerable variability of serum cytokine levels amongst different individuals, the results obtained in this study were generally similar to the associations reported previously, although the results seldom reached statistical significance, probably due to a variety of reasons, some of which are discussed later in this section.

A polymorphism could affect the level of protein production by a gene in several ways. Four of the polymorphisms investigated in this study are located in the promoter region of the gene, and any functional effect is likely to be due to the disruption or creation of transcription factor binding sites: the –308 G allele defines a 10 bp consensus sequence for binding of activator protein-2 (AP-2), a transcription factor which has been shown to repress TNF promoter activity in the Jurkat T-cell line (Takashiba *et al*, 1993; Kroeger and Abraham, 1996); the IL-6 –174 polymorphism lies immediately upstream of a multi-response element which is located between positions –173 and –151 in the IL-6 gene promoter (Ray *et al*, 1990); and the IL-10 polymorphisms at positions –1082 and –592 lie within an ETS-like recognition site (Kube *et al*, 1995), therefore possibly affecting the binding of this transcription factor and ultimately the transcriptional efficiency of the gene. The TNFB polymorphism occurs in intron 2 of the TNF-β gene, and is not located in any known transcription factor recognition sequence, although intron 1 does contain such

Table 5.4 Cytokine serum protein levels of TNF-α (TNF-α -308 and TNFB), IL-6 (IL-6 –174), IL-1β (IL-1β +3953 and IL-1RA), and IL-10 (IL-10 –1082 and –592) in healthy controls classified according to genotype.

Polymorphism	Genotype	Mean ± SE (pg/ml)	Median (pg/ml)	Above Total Group Mean	Above Total Group median
TNF-α -308	GG (n=34)	239 ± 63	143	24%	50%
	GA (n=23)	394 ± 99	179	43%	52%
	AA (n=8)	331 ± 150	90	37%	50%
	GA+AA (n=31)	378 ± 82	142	52%	52%
	Total group (n=65)	305 ± 52	142		
TNFB	B1B1 (n=13)	368 ± 123	138	38%	38%
	B1B2 (n=28)	361 ± 97	153	39%	50%
	B2B2 (n=24)	207 ± 48	189	29%	50%
	B1B1+B1B2 (n=41)	363 ± 76	138	39%	46%
	Total group (n=64)	310 ± 52	160		
IL-6 -174	GG (n=28)	95 ± 28	10	39%	57%
	GC (n=30)	23 ± 10	0	10%	40%
	CC (n=7)	81 ± 30	98	57%	71%
	GC+CC (n=37)	34 ± 10	1	19%	46%
	Total group (n=65)	60 ± 14	3		
IL-1β +3953	CC (n=26)	37 ± 12	6	31%	65%
	CT (n=23)	26 ± 12	4	17%	17%
	TT (n=4)	156 ± 89	103	75%	75%
	CT+CC (n=49)	32 ± 9	5	24%	43%
	CT+TT (n=27)	45 ± 18	4	26%	26%
	Total group (n=53)	42 ± 11	5		
IL-1RN	A1A1 (n=30)	38 ± 15	6	23%	40%
	A1A2 (n=18)	47 ± 20	6	28%	56%
	A2A2 (n=4)	29 ± 16	26	50%	50%
	A1A2+A2A2 (n=22)	44 ± 17	6	32%	55%
	Total group (n=52)	40 ± 11	5		
IL-10 -1082	GG (n=16)	28 ± 5	22	50%	69%
	GA (n=27)	18 ± 2	16	37%	41%
	AA (n=11)	25 ± 4	22	54%	54%
	GA+AA (n=38)	20 ± 2	18	42%	45%
	Total group (n=54)	22 ± 2	20		
IL-10 -592	CC (n=38)	23 ± 3	19	42%	42
	CA (n=15)	21 ± 3	21	33%	47%
	AA (n=2)	33 ± 7	33	100%	100%
	CA+AA (n=17)	23 ± 3	22	47%	53%
	Total group (n=55)	23 ± 2	21		

SE = standard error; n = number of individuals.

sequences (Messer *et al*, 1991). However, this polymorphism is linked to a second polymorphism in the TNF-β gene which leads to an amino acid variation at position 26 (exon 2), where asparagine is associated with the B1 allele and threonine with the B2 allele (Messer *et al*, 1991). The biological significance of this amino acid change has not been determined. The IL-1RN polymorphism is located in intron 2 of the IL-1RN gene. Since individuals with different numbers of the 86bp tandem repeat may have different numbers of potential protein binding sites for transcription factors, it is thought that the polymorphism could be functional as a result. Each tandem repeat contains at least three motifs for DNA-binding proteins, although the possible functional effect of transcription factors binding to sites in introns close to the promoter is not clear (Danis *et al*, 1995). The IL-1β +3953 polymorphism occurs in exon 5 of the IL-1β gene, however, it has been found that this point mutation does not affect the bioreactivity of IL-1β. Thus the mechanism of any effect of this polymorphism on quantitative differences in IL-1β expression levels is not clear (Pociot *et al*, 1992).

In our study, levels of TNF-α serum protein were compared to genotype of the two TNF polymorphisms. Our results correlated with most previous studies (Wilson *et al*, 1997; Kroeger *et al*, 1997; Warzocha *et al*, 1998; Louis *et al*, 1998; Maurer *et al*, 1999) in finding higher levels of TNF-α to be linked to the TNF-α -308 A allele. The effect of the TNFB polymorphism is less clear, and we found the B1 allele to be linked to higher TNF-α levels, although this linkage was not statistically significant unlike the -308 results. This could indicate that the TNFB polymorphism does not have a true functional effect but is linked to other markers that do, possibly the TNF-α -308 polymorphism, as discussed later in this section. For both these TNF polymorphisms, when looking at median values instead of mean values, the associations with genotype were not seen. This is probably because several individuals had TNF-α levels of 1000 pg/ml and above, whereas most readings were between 0 and 500 pg/ml (Figure 5.1). The IL-6 –174 G allele was found to be associated with higher IL-6 levels, as has previously been reported (Fishman *et al*, 1998; Burzotta *et al*, 2001). The CC group was linked to higher IL-6 levels than the GC group, but as there were only seven individuals with the CC genotype, the GC and CC individuals were placed in one group for statistical analysis. The IL-1β +3953 TT group included only 4 individuals but was associated with very high IL-1β, as previously reported (Pociot *et al*, 1992). Only a slight association of the T allele with increased IL-1β levels remained when the CT and TT individuals were grouped. We found no significant change in IL-1β levels

when comparing to IL-1RN VNTR alleles, and it would be interesting to look at the effect of this polymorphism on IL-1Ra levels. The IL-10 –1082 G allele has previously been associated with increased IL-10 levels compared to the A allele (Turner *et al*, 1997), a trend seen in our study, with GG individuals exhibiting an increased, though not statistically significant, level of serum IL-10 compared to GA and AA individuals. The IL-10 –592 polymorphism was not associated with IL-10 levels.

Although there are many studies investigating the functional effect of the polymorphisms studied in this chapter, it appears that in many cases no absolute consensus has yet been reached. This is probably due to a variety of factors including the variation of *in vivo* cytokine levels as a result of age, body mass index (BMI), gender, smoking status, recent viral or bacterial infection, activity level/food intake before blood sample donation, and whether samples were from healthy controls or from patient groups. Previous studies include reporter gene experiments which compare promoter activity of the two alleles in response to a stimulus (Fishman *et al*, 1998; Wilson *et al*, 1997; Kroeger *et al*, 1997), measurement of plasma cytokine levels (Fishman *et al*, 1998; Mira *et al*, 1999), and measurement of the *in vitro* cytokine expression of whole blood, PBMCs, monocytes, and other isolated cell types, the latter two methods involving the comparison of genotypes (Messer *et al*, 1991; Pociot *et al*, 1991; Pociot *et al*, 1993). An alternative to measuring cytokine resting levels is to measure levels in whole blood or cell culture in response to stimulation. In these experiments, a range of stimulants have been used, including LPS, PHA, PPD, IL-1, and Con A, most protocols varying in incubation period and stimulant concentration (Louis *et al*, 1998; Pociot *et al*, 1991; Pociot *et al*, 1993; Turner *et al*, 1997). Some previous studies have compared homozygotes, excluding heterozygotes from the equation (Messer *et al*, 1991), whereas, more often, in the case of a low frequency allele, homozygotes and heterozygotes are grouped and compared to homozygotes for the high frequency allele (Turner *et al*, 1997; Louis *et al*, 1998). The possibility of results obtained by chance due to factors such as small population numbers, concentration of stimulant, and culture conditions must be considered, although this possibility decreases with an increase in the number of studies reporting an association of one allele or genotype with greater or lesser levels of the relevant cytokine.

Our study investigated the relationship between cytokine levels and genotype in a random group of control individuals. A more relevant study could be to investigate the cytokine protein levels in individuals with various autoimmune/inflammatory diseases. Genotype-

associated fluctuations in cytokine levels may be involved in causing greater susceptibility to inflammatory disease or causing more severe disease once a patient has developed the disease. Thus the association between a genotype and increased cytokine levels might be clearer when an individual has been exposed to an inflammatory stimulus, for example Koss *et al* found a higher association of increased IL-10 levels with IL-10 -1082 genotype in Crohns' disease ($p=0.005$) compared to controls ($p=0.015$) (Koss *et al*, 2000).

Almost all studies on the effect of the TNF-α -308 polymorphism show that the rarer A allele is associated with higher levels of TNF-α production. However, studies on the effect of the TNFB polymorphism are more divergent. The TNF-α and TNF-β genes are located together in the TNF locus in the MHC region, and so the polymorphisms discussed here are linked. It is therefore possible that any results obtained for either of these polymorphisms is actually due to linkage with the other polymorphism or else linkage of both of these polymorphisms with another nearby marker. We found that the –308 A allele is linked to the TNFB1 allele, with the finding that AA individuals in the control group always have the TNFB1B1 genotype, although the opposite is not the case. It is therefore difficult to reconcile how the B2 allele could be linked to higher levels of TNF-α, as has been reported (Pociot *et al*, 1991; Pociot *et al*, 1993; Stüber *et al*, 1996; Majetschak *et al*, 1999), if the TNF-α -308 A allele is also associated with higher TNF-α levels, since the B2 allele occurs more frequently with the –308 G allele. The finding in our study that the –308 A allele and TNFB1 alleles are both associated with higher TNF-α levels ties in with the fact that these two alleles are linked. Similarly, the IL-10 –592 genotype is in close proximity to, and thus in linkage disequilibrium with, the IL-10 –1082 polymorphism. In our control study group, individuals carrying the –1082 GG genotype always carry the –592 CC genotype, although the opposite is not true. However, this linkage is not reflected in the IL-10 protein results obtained for the two polymorphisms perhaps due to the small differences seen in IL-10 protein levels overall.

To conclude, although generally not statistically significant, the results obtained in this study follow the trend of previous studies, in associating certain alleles or genotypes with higher levels of the relevant cytokine. However, factors such as the inter-individual variation in serum cytokine levels as a result of age, gender, BMI, etc, may have played a role in decreasing the potential to fully elucidate the functionality of the polymorphisms under investigation. With this in mind, reporter gene studies or *in vitro* cell culture studies of isolated human cells are potentially a more robust experimental model for this type of

investigation. However, due to the large variety of protocols used in different studies, the current findings for the functionality of these cytokine polymorphisms are inconclusive. A possible solution to this problem was proposed by Warle *et al*, who advanced the idea of setting up a multicenter workshop in which the relationship between certain cytokine gene polymorphisms and *in vitro* cytokine production could be analysed using an identical *in vitro* cell culture system and study population (Warle *et al*, 2003).

5.4 MENINGOCOCCAL SEPSIS

5.4.1 Introduction

First recorded by Vieusseaux in 1805 (Vieusseaux, 1805), meningococcal disease is a worldwide public health problem and, when associated with purpura fulminans and haemodynamic deterioration, mortality rates of 20 to 30% have been recorded (Pearson *et al*, 2000; Kornelisse *et al*, 1997). In those patients who survive the acute phase of the severe illness, mutilating complications and end-organ failure are common (Havens *et al*, 1989; Giraud *et al*, 1991). Hyperendemic invasive meningococcal disease (IMD) in Ireland, which currently has the highest incidence of laboratory confirmed meningococcal disease in Europe (Noah and Henderson, 2001), continues to cause concern as a result of the unpredictability of the disease.

The pathophysiology of severe meningococcaemia is becoming increasingly clear. Meningococcal endotoxin, LPS, is now understood to be crucial in the pathogenesis of the disease. This microbial constituent, in a dose-dependent manner, activates the haemostatic cascades of procoagulation, anticoagulation, fibrinolysis, and the complement and kallikrein/kinin systems, together with inflammatory mediator systems such as the cytokine network (Brandtzaeg *et al*, 1989). The net procoagulant effect and the generation of proinflammatory cytokines result in widespread microvascular thrombosis, tissue toxicity and injury, and end organ failure (Smith and White, 1999).

High circulating plasma levels of IL-6 have been associated with poor outcome in patients with meningococcal shock (van Deuren *et al*, 1995; Waage *et al*, 1989), sepsis (Hack *et al*, 1989; Takala *et al*, 1999), melioidosis (Simpson *et al*, 2000), and in neonatal sepsis and other neonatal septic conditions (Harris *et al*, 1994). TNF-α is widely reported to be a pivotal mediator of sepsis, septic shock, and multiple organ failure, and high circulating plasma levels of TNF-α have been found to correlate with poor outcome in patients with meningococcal shock (van Deuren *et al*, 1995; Waage *et al*, 1987; Westendorp *et al*, 1995), and in patients with other forms of sepsis (Debets *et al*, 1989; Girardin *et al*, 1988). As with TNF-α and IL-6, levels of IL-1β are associated with outcome in meningococcal disease (van Deuren *et al*, 1995; Waage *et al*, 1989; Girardin *et al*, 1988). IL-10, an important suppressor of the inflammatory response, down-regulates endotoxin stimulated monocyte synthesis of pro-inflammatory cytokines such as IL-1, TNF-α, IL-6, IL-8, and

IL-12, and up-regulates the synthesis of IL-1Ra (de Waal Malefyt *et al*, 1991; Fiorentino *et al*, 1991).

A number of cytokine gene polymorphisms have been described in association with altered constitutive and inducible levels of cytokines (Bidwell *et al*, 1999), and this provides a rationale for their potential role in meningococcal disease severity. Although there is some conflicting evidence for the functionality of these polymorphisms, we postulated that polymorphisms which influence variations in cytokine levels could have an effect on the inflammatory response to invasive infection with *Neisseria meningitidis*. Given the observations that plasma levels of the pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 are elevated in this disease, and that higher levels directly correlate with increased severity and fatality, we investigated whether polymorphism status in pro-inflammatory and anti-inflammatory genes is associated with disease severity and outcome in meningococcal septic shock. We therefore studied the genotype frequencies of seven such polymorphisms in a group of patients with meningococcal disease and a control population, in order to determine if any polymorphism is associated with disease susceptibility, severity, or outcome.

5.4.2 Subjects and Methods

Controls

Blood samples were collected from 389 blood donors (Section 5.2.1).

Meningococcal Disease Patients

The Irish Meningococcal and Meningitis Reference Laboratory (IMMRL) at The Children's University Hospital, Temple Street, Dublin, provides a national service for non-culture diagnosis of IMD by polymerase chain reaction (PCR) analysis for the *citrA* gene (specific to *N. meningitidis*) in blood, cerebrospinal fluid and samples from other sterile sites. Residual bloods from samples taken for routine PCR are stored in the IMMRL at -70°C. Ethical approval for this study using residual blood samples submitted for PCR from patients in the IMMRL database, was obtained from The Children's University Hospital on condition that all samples be irreversibly unlinked from patient identifiers and clinical details prior to analysis.

The study cohort was derived from two sources: children identified from the database between January 1997 and February 2000, who presented to one of two tertiary referral paediatric hospitals in Dublin (The Children's University Hospital, Temple Street, and Our Lady's Hospital for Sick Children, Crumlin,) and secondly patients on the database known to have died in other hospitals, and on whom such samples were available. The medical records of study children were reviewed and clinical and laboratory data collected retrospectively, with patient identification details known to only one investigator, Dr Mary C Healy, prior to unlinking samples. Meningococcal disease treatment protocols were similar in both hospitals. Complete data required for a Glasgow Meningococcal Sepsis Prognostic Score (GMSPS), or PRISM score, were not available on all patients. A scoring system based on prognostic factors independently demonstrated as being predictive of severe illness and poor outcome in IMD, for which complete data were available on all children, was designed (Lodder *et al*, 1996; Flaegstad *et al*, 1995; McManus and Churchwell, 1993; Algren *et al*, 1993; Tesoro and Selbst, 1991; Gedde-Dahl *et al*, 1990; Arevalo *et al*, 1989; Wong *et al*, 1989; Emparanza *et al*, 1988; Gardlund, 1986; Leclerc *et al*, 1985; Anderson, 1978; Kornelisse *et al*, 1997; Gamper *et al*, 2001; Mertens *et al*, 1999; Sorensen *et al*, 1998; Malley *et al*, 1998; Barquet *et al*, 1999; Anderson *et al*, 1997; Luaces Cubells *et al*, 1997; Malley *et al*, 1996; Olivares *et al*, 1993). Additional therapies (e.g. ventilation, inotropes, protein C, haemofiltration) and clinical indicators of severity of illness (e.g. prolonged intensive care unit [ICU] stay or fever) were also included for scoring purposes. Independent verification of the scoring system was obtained from paediatricians not involved in this study, who are experienced in the care of children with IMD.

Patients were divided into two groups for statistical analysis. The first analysis involved dividing the cohort into two groups based on clinical severity of disease. Group 1 comprised patients with mild illness who recovered without sequelae. Group 2 comprised those with many poor prognostic factors, survivors with permanent sequelae (permanent scarring, neurological deficit, sensorineural hearing loss, renal failure, amputation) and all deaths. In order to determine whether the presence of a particular polymorphism was associated with mortality, the total population (n=183) was then divided into those who survived (n=158) and those who had fatal infection (non-survivors, n=25).

Meningococcal Sepsis Samples

DNA was isolated from the meningococcal sepsis blood samples (Section 5.2.2), and PCR and RFLP analysis was performed in order to genotype samples for seven cytokine gene polymorphisms (Sections 5.2.3-5.2.5).

5.4.3 Results

Control and Patient Groups

One hundred and ninety three samples on patients with IMD were collected (110 mild, 83 severe). Group B meningococcus accounted for 78% of meningococcal infections and group C accounted for the remainder. Characteristics of patients with IMD presenting to one of two paediatric hospitals and on whom clinical details were available prior to unlinking samples are shown in Table 5.5. Clinical details were unavailable for 22 patients (11%) who died in other hospitals. For all seven cytokine polymorphisms, 389 control samples and 183 IMD patient samples were genotyped. For analysis, the IMD group was divided into mild disease (n=107) and severe disease (n=76) groups. For further analysis, this group was also divided into survivor (n=158) and non-survivor (n=25) groups. Therefore, three separate comparisons were made in this study. Firstly, the IMD group was compared to the control group for each of the seven polymorphisms. The second comparison was made between the mild and severe disease groups, whilst the final comparison was between the survivor and non-survivor groups for each polymorphism.

IL-6 G-174C Polymorphism

Raw data, genotype frequencies, and allele frequencies obtained for this polymorphism in the control and IMD populations are listed in Table 5.6. Genotype frequencies did not differ significantly between the IMD patient group and the control group for the IL-6 promoter polymorphism (p=0.7). Within the IMD population, the GG genotype occurred at a frequency of 41% in the severe disease group, compared to 26% in the mild disease group (p=0.037). When looking at mortality, the GG genotype was more common amongst those patients that died (52%) compared to survivors (29%) (p=0.023). Similarly, carriers of the G allele were significantly more common, and carriers of the C allele were significantly less common, in the group of patients that died than in the group of patients with mild disease (p=0.03). On the basis of these gene frequencies, patients with IMD who carry the GG genotype had a significantly increased risk for death (odds ratio 2.64 [95% CI 1.12-6.22]) compared with the two other genotypes.

Table 5.5 Clinical characteristics of children with IMD. All values are quoted as median (range) for continuous variables and frequency (percent) for categorical values.

Clinical Details	Mild (n=110)	Severe (n=61)[a]
Age (months)	23.5 (2-182)	19 (3-179)
Bacteraemia	79 (72)	41 (67)
Meningitis	8 (7)	0 (0)
Bacteraemia and meningitis	23 (21)	20 (33)
Clinically shocked on admission [b]	71 (64)	52 (85)
Volume of resuscitation fluid in first hour (ml/kg)	20 (0-90)	40 (0-80)
Admission to ICU	44 (40)	54 (89)
Ventilated	1 (1)	36 (59)
Inotropes	1 (1)	19 (31)
Haemofiltration or Protein C	0 (0)	14 (23)
Duration of ICU (hours)	0 (0-48)	58 (0-1488)
White cell count $< 5 \times 10^9$/L	5 (5)	18 (33)
Platelets $< 150 \times 10^9$/L	6 (5)	11 (18)
Coagulopathy [c]	56 (51)	47 (77)

n = number of individuals.

[a] Clinical details unavailable on 22 patients who died in other hospitals.

[b] tachycardia, capillary refill ≥ three seconds and administration of >10 mls/kg fluid bolus in the emergency room, with or without hypotension.

[c] prolonged prothrombin time (PT) or activated partial thromboplastin time (APTT).

Table 5.6 Raw data, genotype or allele frequencies (in brackets), and χ^2 p values for the IL-6 -174 and IL-10 –1082 polymorphisms in patients with meningococcal disease and in a control population. The patient group is further divided into groups of disease severity, and groups of survivors and non-survivors.

Genotype or Allele	BTSB Controls	Menin-gococcal sepsis	Mild	Severe	p value[a]	Survivors	Non-survivors	p value[b]	p value[c]
	n=389	n=183	n=107	n=76		n=158	n=25		
IL-6 –174 GG	123 (32)	59 (32)	28 (26)	31 (41)	0.037	46 (29)	13 (52)	0.023	0.012
IL-6 –174 GC	198 (51)	97 (53)	62 (58)	35 (46)		87 (55)	10 (40)		
IL-6 –174 CC	68 (17)	27 (15)	17 (16)	10 (13)		25 (16)	2 (8)		
IL-6 –174 G	444 (57)	215 (59)	118 (55)	97 (64)	0.097	179 (57)	36 (72)	0.04	0.03
IL-6 –174 C	334 (43)	151 (41)	96 (45)	55 (36)		137 (43)	14 (28)		
IL-10 –1082 GG	123 (32)	52 (29)	34 (32)	18 (24)		44 (28)	8 (32)		
IL-10 –1082 GA	180 (46)	95 (52)	59 (55)	36 (47)		85 (54)	10 (40)		
IL-10 –1082 AA	86 (22)	36 (19)	14 (13)	22 (29)	0.0078	29 (18)	7 (28)	0.25	0.067
IL-10 –1082 G	426 (55)	199 (54)	127 (59)	72 (47)		173 (55)	26 (52)		
IL-10 –1082 A	352 (45)	167 (46)	87 (41)	80 (53)	0.0234	143 (45)	24 (48)	0.71	0.34

[a] χ^2 p value for genotypes or alleles: mild vs severe.
[b] χ^2 p value for genotypes or alleles: survivors vs non-survivors.
[c] χ^2 p value for genotypes or alleles: mild vs non-survivors.
n = number of individuals.

IL-10 –1082 Polymorphism

Genotype and allele frequencies for this polymorphism are shown in Table 5.6. Comparison of allele and genotype frequencies did not reveal any significant difference between patients and controls. However, when patients were subgrouped according to disease severity, the AA genotype was found to occur at a high frequency in the group of patients with severe disease and at a low frequency in those suffering from mild disease (29% vs 13%, p=0.0078, odds ratio 2.7 [95% CI 2.3-3.6]), and, although not statistically significant, a similar trend for survivors vs non-survivors was seen (28% vs 18%, p=0.25).

IL-1RN VNTR

Frequencies of IL-1RN genotypes in the IMD population and in the control population are shown in Table 5.7. For the IL-1RN VNTR, the only statistically significant finding amongst the groups of disease severity was found to occur in the fatal group, where the A1A2 genotype was significantly lower (20% vs 37-41%, p=0.0433), and A1A1 higher, than in any of the other groups. The frequency of the A2A2 genotype was significantly lower in the control group compared to the IMD group (p=0.0326).

IL-1β +3953, IL-10 –592, TNFB, TNF-α -308

Genotype and allele frequencies were not significantly different amongst the groups of disease severity for these polymorphisms, or when comparing the disease and control groups (Table 5.7), despite some variation in genotype frequencies amongst disease groups.

Multi-polymorphism Analysis: IL-6 -174, IL-10 –1082, IL-1RN VNTR

Details of analysis of combinations of genotypes are shown in Table 5.8. Combinations of genotypes identified as potentially associated with IMD severity and survival by univariate analysis (IL-6 –174 GG, IL-10 –1082 AA, and IL-1RN 11) were investigated. Higher frequencies of individuals carrying combinations of high risk genotypes were seen in the severe disease group compared to the mild group, and in the non-survivor group compared to the survivor group, although not all had significant χ^2 P values (Table 5.8). In order to assess the simultaneous presence of all three risk variants, nominal logistic regression was used to test the combined effect of carriage of the IL-6 –174 GG, IL-10 –1082 AA, and IL-1RN A1A1 genotypes together. For mild versus severe disease, it was found that these three factors are not significantly associated with disease severity (p=0.08). The same

Table 5.7 Raw data and genotype frequencies (in brackets) of five cytokine polymorphisms in a control population and in a meningococcal sepsis population. The patient group is further divided into groups of disease severity, and groups of survivors and non-survivors.

Polymorphism	Genotype	BTSB Controls n=389	Meningo-coccal sepsis n=183	Mild n=107	Severe n=76	Survivors n=158	Non-survivors n=25
TNFB	B1B1	52 (13)	32 (17)	20 (19)	12 (16)	29 (18)	3 (12)
	B1B2	205 (53)	93 (51)	53 (49)	40 (53)	79 (50)	14 (56)
	B2B2	132 (34)	58 (32)	34 (32)	24 (31)	50 (32)	8 (32)
TNF-α -308	GG	233 (60)	100 (55)	59 (55)	41 (54)	85 (54)	14 (56)
	GA	140 (36)	75 (41)	43 (40)	32 (42)	66 (42)	10 (40)
	AA	16 (4)	8 (4)	5 (5)	3 (4)	7 (4)	1 (4)
IL-10 –592	CC	235 (60)	125 (68)	79 (74)	46 (60)	109 (69)	16 (64)
	CA	139 (36)	52 (29)	24 (22)	28 (37)	44 (28)	8 (32)
	AA	15 (4)	6 (3)	4 (4)	2 (3)	5 (3)	1 (4)
IL-1β +3953	CC	240 (62)	113 (62)	69 (64)	44 (58)	100 (63)	13 (52)
	CT	125 (32)	65 (35)	33 (31)	32 (42)	53 (34)	12 (48)
	TT	24 (6)	5 (3)	5 (5)	0 (0)	5 (3)	0 (0)
IL-1RN	A1A1	183 (47)	83 (45)	49 (46)	34 (45)	68 (43)	15 (60)
	A1A2	159 (41)	70 (38)	42 (39)	28 (37)	65 (41)[a]	5 (20)[a]
	A2A2	31 (8)[b]	25 (14)[b]	13 (12)	12 (16)	21 (13)	4 (16)
	A1A3	15 (4)	5 (3)	3 (3)	2 (2)	4 (3)	1 (4)
	A2A3	1 (0.2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a] χ^2 p value for survivors vs non-survivors=0.0433.
[b] χ^2 p value for controls vs meningococcal disease=0.033.
n = number of individuals.

Table 5.8 Raw data, frequencies (in brackets), and p values for the carriage of combinations of the IL-6 –174 GG, IL-10 –1082 AA, and IL-1RN VNTR A1A1 genotypes in patients with meningococcal disease divided into groups of disease severity and groups of survivors and non-survivors.

Genotype Combination	Mild	Severe	p value[a]	p value[b]	Survivors	Non-survivors	p value[c]	p value[d]
	n=107	n=76			n=158	n=25		
IL-6 –174 GG, IL-10 -1082 AA, IL-1RN VNTR A1A1	1 (1)	4 (5)	0.037	0.0816	2 (1)	3 (12)	0.0022	0.1105
IL-6 –174 GG, IL-10 -1082 AA	3 (3)	10 (13)	0.016	0.0094	9 (6)	4 (16)	0.06	0.116
IL-6 –174 GG, IL-1RN VNTR A1A1	14 (13)	12 (16)	0.6	0.15	17 (11)	9 (36)	0.0008	0.026
IL-10 -1082 AA, IL-1RN VNTR A1A1	6 (6)	10 (13)	0.075	0.07	11 (7)	5 (20)	0.032	0.2

[a] χ^2 p value, mild vs severe; [b] nominal logistic regression p value, mild vs severe; [c] χ^2 p value, survivors vs non-survivors; [d] nominal logistic regression p value, survivors vs non-survivors.
n = number of individuals.

analysis looking at survival found these three factors to have even less of an influence (p=0.11). Testing the effect of carriage of two genotypes, significant P values were obtained for IL-6 –174 GG with IL-10 –1084 AA for mild versus severe disease; and for the IL-1RN VNTR A1A1 genotype with both the IL-6 –174 GG and the IL-10 –1082 AA genotypes for survival, although the IL-1RN A1A1 and IL-6 GG combination was more significant (χ^2 p=0.0008) (Table 5.8).

5.4.4 Discussion

Meningococcal disease severity varies widely, from bacteraemia, through meningitis, to overwhelming septicaemia, although it is unclear why some patients develop mild manifestations of invasive meningococcal infection, whilst at the other end of the spectrum some individuals rapidly develop fulminant septicaemia characterised by extensive microvascular thrombosis, consumptive coagulopathy, multi-organ dysfunction, and a high risk of mortality and mutilating complications, particularly in those patients who develop septic shock. Meningococcal septicaemia results from the release of bacterial endotoxin into the bloodstream, with the subsequent activation of systemic inflammation and coagulation cascades, and inhibition of fibrinolysis. Many of the sequelae associated with endotoxaemic-induced shock result from excessive production of the cytokine mediators TNF-α, IL-1β, and IL-6 from LPS-activated monocytes. Genomic polymorphisms within the genes encoding these endogenous mediators may affect the extent of an individual's inflammatory response, and hence could contribute to morbidity and mortality patterns in patients with severe sepsis. We therefore genotyped a population of patients with meningococcal disease and a large control population for polymorphisms in seven cytokine genes in order to determine if certain genotypes are associated with disease susceptibility, severity, or survival.

The IL-6 –174 G allele has previously been associated with increased IL-6 levels (Fishman *et al*, 1998; Burzotta *et al*, 2001; Villuendas *et al*, 2002), although the association may be age and sex related (Olivieri *et al*, 2002; Kilpinen *et al*, 2001), and no association between this polymorphism and IL-6 levels has also been reported (Nauck *et al*, 2002). We found an association between homozygous carriage of the IL-6 –174 G allele and poor outcome in meningococcal disease. Higher IL-6 levels have been found to be strongly associated with mortality in meningococcal sepsis (van Deuren *et al*, 1995; Waage *et al*, 1989), and other forms of sepsis (Takala *et al*, 1999; Simpson *et al*, 2000). No significant difference in

frequency of genotypes or alleles was found between the group with meningococcal disease and the control population. These findings suggest that while the IL-6 –174 G allele does not increase the risk of an individual acquiring invasive meningococcal infection, its presence will, however, have some influence on whether or not the individual develops severe disease and multiple organ dysfunction during the systemic inflammatory response to *N. meningitides*. The mechanism of this effect of IL-6 is unclear. However, IL-6 is generally regarded as pro-inflammatory and therefore homozygous carriage of the -174 G allele, which is associated with higher plasma IL-6 levels, may cause a more pro-inflammatory profile in *N. meningitides* infected individuals, leading to the cytokine driven multiorgan failure that is associated with mortality in the disease. IL-6 also has pro-coagulant properties (Stouthard *et al*, 1996), and thus –174 G homozygous individuals may have a greater thrombin generation potential than carriers of other genotypes, possibly contributing to the purpura fulminans and DIC that is associated with meningococcaemia.

Meningococcal septicaemia is associated with a pro-inflammatory state, and IL-10 is believed to play an important role in regulating any such response. The finding that the AA genotype of the IL-10 -1082 polymorphism is at a significantly higher frequency in the severe meningococcal disease group compared to the other groups of disease severity, associates a genotype that has been linked to lower levels of the anti-inflammatory cytokine IL-10 (Turner *et al*, 1997) with more severe disease. This observation correlates with the IL-6 polymorphism finding, and the net result relates both a genotype thought to be associated with higher levels of a pro-inflammatory cytokine (IL-6 -174 GG) and one associated with lower levels of an anti-inflammatory cytokine (IL-10 -1082 AA), an overall pro-inflammatory profile, to more severe meningococcal disease. The non-survivors group was not significantly associated with the IL-10 -1082 genotype, despite increased levels of the IL-10 –1082 AA genotype, which could be due to the relatively small size of this group (n=25). Although not statistically significant, the IL-10 -592 polymorphism had varying genotype frequencies, especially between the mild and severe disease groups, which could be due to linkage with the IL-10 -1082 polymorphism, since the two polymorphisms are located in close proximity in the IL-10 gene.

Production of IL-1β, along with TNF-α, is stimulated by bacterial toxins or inflammation early in the acute phase response, causing the subsequent expression of a variety of cytokine and transcription factor genes. In this study, no association was found between the IL-1β +3953 polymorphism and meningococcal disease. The balance between IL-1β

and IL-1Ra is of importance in normal and disease states, as the margin between beneficial levels of IL-1β and toxicity is very narrow in humans. Despite the expectation that a low IL-1Ra plasma concentration might aggravate the morbidity of sepsis by allowing increased pro-inflammatory activity to occur, it appears that a co-ordinated and balanced IL-1/IL-1Ra ratio in the circulation is more important in the course of systemic inflammatory challenges than an overall pro- or anti-inflammatory response, as seen by recombinant IL-1Ra therapy clinical trial results (Fisher *et al*, 1994), and the effects of IL-1Ra on mouse and rat sepsis models (Hirsch *et al*, 1996; Mancilla *et al*, 1993). Allele 2 of the IL-1RN VNTR, linked to both increased and decreased levels of IL-1Ra in different studies (Danis *et al*, 1995; Tountas *et al*, 1999), and thus possibly increased anti-inflammatory or pro-inflammatory activity, has been associated with susceptibility to sepsis (Fang *et al*, 1999). Our finding of a significantly lower level of the A2A2 genotype in controls compared to the meningococcal group as a whole correlates with this study. In contrast, our study also detected a lower frequency of the A1A2 genotype, and higher frequency of the A1A1 genotype, in the group of patients that died from meningococcal disease compared to those that survived, indicating that carriage of the A1 allele may predispose a poor outcome in meningococcal disease.

As the purpose of this study is to characterise a genetic risk profile for IMD, the simultaneous presence of the identified risk variants in all individuals was assessed. Although carriers of all three risk factors thus far identified, IL-6 –174 GG, IL-10 –1082 AA, and IL-1RN VNTR A1A1, were more common in the severe or non-survivor groups compared to mild or survivor groups (Table 5.8), these associations were not notably more significant than when looking at the risk factors alone. Nominal logistic regression analysis of carriage of two higher risk genotypes revealed a significant association for carriage of IL-6 –174 GG and IL-10 –1082 AA when comparing mild to severe disease; and for carriage of the IL-6 –174 GG and IL-1RN VNTR A1A1 when comparing survivors to non-survivors (Table 5.8). However, despite the significant associations obtained from multivariate analysis, the fact that this analysis did not show greatly increased significance compared to univariate analysis leads to the conclusion that these three factors are associated with IMD severity and survival independently of each other, and IMD patients carrying all three genotypes are not at a significantly greater risk of more severe IMD or death than those carrying just one of these factors alone.

Both the TNF-α –308 and TNFB polymorphisms have been associated with variations in TNF-α levels (Fang *et al*, 1999; Stüber *et al*, 1996; Majetschak *et al*, 1999), and may therefore have an effect on IMD severity and outcome. However, we found no association between alleles of either the TNF-α or TNF-β polymorphisms and disease outcome or severity. This contrasts studies linking severe sepsis outcome and TNFB genotype (Stüber *et al*, 1996; Majetschak *et al*, 1999), and the TNF-α -308 polymorphism with outcome in septic shock and meningococcal disease respectively (Mira *et al*, 1999; Nadel *et al*, 1996). However, our findings concur with those of another study in which it was reported that there is no association between the TNF-α -308 polymorphism and severe sepsis (Stüber *et al*, 1995). A number of factors could be responsible for the discrepancy between the findings in these different studies. Populations from different countries may have different risk factors for severe meningococcal disease, our results indicating that, although the two TNF polymorphisms are not risk factors in the Irish population, the IL-6, IL-10, and IL-1RN polymorphisms do appear to be risk factors. The size of the study populations must also be taken into consideration, since larger study populations decrease the likelihood of spurious results. The sizes of the sepsis study populations mentioned above (n=40, 110, 89, and 98) are slightly smaller than the meningococcal disease population investigated in this study (n=183). The method of scoring disease severity is also important, and definite comparisons can not be made between studies unless the same criteria for dividing groups into disease severity categories have been used. Even the traditional method of dividing the disease group into survivors and non-survivors can not be fully relied on as there will be a higher mortality rate in cases where there is a greater delay before hospitalisation and treatment, as may more often be the case in adults compared to children. Patient care can also have a bearing on severity and outcome of disease. The rapidity of treatment and the type of treatment can vary from case to case, which will automatically have an effect on outcome. Both these factors are likely to differ in different geographical areas. The causative organism may lead to slightly different inflammatory responses, and infections caused by different bacteria may have varying associations with polymorphisms. Finally, it has been reported that gender may also have an effect on whether a patient's genotype is associated with outcome. A study of the TNFB allele in patients with severe sepsis found that mortality was increased for males homozygous for the B2 allele, but there was no difference in survival rate in females (Schroder *et al*, 2000). Gender was not taken into account in our current study (due to the retrospective nature of the study and restrictions imposed by the hospital Ethics Committee) or in three of the other severe sepsis studies (Stüber *et al*, 1996; Nadel *et al*, 1996; Mira *et al*, 1999). Majetschak *et al* (Majetschak *et*

al, 1999) found an association between B2 homozygosity and disease severity, however their study group was predominantly male (70%). This association may not have been found if the study group had been predominantly female.

Our study had some limitations and potential sources of bias. Some patients who died presented to other centres and clinical data were unavailable. The retrospective study design and the requirement of the Ethics Committee that all samples be completely and irreversibly unlinked from all patient details prior to analysis rendered us unable to analyse polymorphisms associations with factors such as age and gender. This study should therefore be viewed as a preliminary exploratory study. It must be noted that the associations found in this study, although statistically significant, were not highly so, and thus further investigations are required in this area. Thus, the presence of the IL-6 –174 GG and IL-10 -1082 AA genotypes, along with the IL-1RN VNTR association noted in this study, are likely to be only a part of the genomic polymorphic risk profile for severe meningococcal sepsis. Genes encoding proteins outside the cytokine network but closely linked with the systemic inflammatory response, such as those involved in procoagulation, anticoagulation, fibrinolysis, complement, and kallikrein/kinin systems are potential candidates for further study. For example, plasminogen activator inhibitor-1 (PAI-1) levels are approximately twice as high in non-survivors of meningococcal disease compared to survivors, whilst circulating PAI-1 levels are significantly higher in patients with severe meningococcal disease in comparison with those with milder disease (Kornelisse *et al*, 1996). Increased PAI-1 levels are associated with carriage of the 4G4G genotype of a 4G5G polymorphism within the promoter region of the PAI-1 gene, and carriage of this high PAI-1 producing genotype is associated with an adverse outcome in severe meningococcal infection (Hermans *et al*, 1999; Westendorp *et al*, 1999).

Why some individuals develop a more severe form of meningococcaemia is not fully understood but is undoubtedly multifactorial, and we believe, as in other forms of sepsis, that no single cause and effect paradigm exists. We do know that there is a strong correlation between the severity of the acquired protein C deficiency that is usually seen in this disease, the extent of the thrombotic skin lesions, and a negative clinical outcome (Fijnvandraat *et al*, 1995). Likewise, it is now understood that certain genetic determinants of an individual's systemic inflammatory response may also influence outcome in these patients. The finding of the present study, that polymorphisms in the IL-6, IL-10, and IL-1RN genes are associated with disease outcome in meningococcaemia, will hopefully

generate further insights into the pathophysiology of sepsis that will encourage future clinical investigations into different biological interventions that will ultimately lead to the availability of effective anti-sepsis therapies.

5.5 PSORIATIC ARTHRITIS

5.5.1 Introduction

Psoriatic arthritis (PsA) is an inflammatory arthritis that occurs in approximately 5-17% of patients with psoriasis and is reported to have an estimated prevalence of approximately 0.3% in the general population (Kay *et al*, 1999). PsA was initially believed to be the coincident occurrence of two common diseases (psoriasis and rheumatoid arthritis). However, based on epidemiological and clinical studies, PsA is now considered to be a unique arthropathy that is characterised by distinctive features such as nail dystrophy, seronegativity for rheumatoid factor, distal interphalangeal joint involvement, and dactylitis (O'Neill and Silman, 1994; Dougados *et al*, 1991). PsA is a heterogenous arthropathy and can range in severity from a mild non-destructive disease to a severe, progressive, erosive arthropathy (Gladman *et al*, 1990; Gladman and Brockbank, 2000). Initial studies suggested that the majority of cases of PsA had a good prognosis with only 5% developing the severe destructive arthritis mutilans (Moll and Wright, 1973b) and 10% having radiological damage (Roberts *et al*, 1976). Subsequent studies have found a higher frequency of radiological damage, with up to 57% of patients with established PsA having erosive arthritis (Torre Alonso *et al*, 1991). Early age of PsA onset, female gender, and the presence of polyarthritis have been associated with a poor prognosis, though the pathophysiological mechanisms underlying poor prognosis PsA are not known.

PsA is characterised by infiltration of the synovial tissue by lymphocytes and macrophages, with prominent new vessel formation (Veale *et al*, 1993). These cells produce pro-inflammatory and anti-inflammatory cytokines which regulate the inflammatory process in the joint (Danning *et al*, 2000). Significantly higher serum and synovial fluid levels of pro-inflammatory (IL-1, IL-2, sIL-2R, IL-6, TNF-α, sTNF R55, sTNF R75) and anti-inflammatory (IL-1Ra, IL-10) cytokines have been found in patients with PsA in comparison to non-inflammatory controls and healthy volunteers (Elkayam *et al*, 2000; Partsch *et al*, 1997; Partsch *et al*, 1998a; Partsch *et al*, 1998b). The Th1 cytokines IL-1β, TNF-α and IL-15 are increased in PsA synovium, though at a lower level than observed in rheumatoid arthritis (RA), while IL-1α and IL-10 are present at similar levels to those seen in RA. The success of novel therapeutic agents such as anti-cytokine antibodies, interleukins, and anti-T-cell therapies, which specifically block cytokine

effects, provide further evidence of the central role of cytokines in inflammation in PsA (Bagel *et al*, 1998; Mease *et al*, 2000).

Through population studies, psoriasis and PsA have been found to be familial, with a multifactorial pattern of inheritance (Steinberg *et al*, 1951; Moll and Wright, 1973a; Rahman *et al*, 1999). Both diseases have associations with certain human leukocyte antigen (HLA) haplotypes (Woodrow and Ilchysyn, 1985) located in the MHC region on chromosome 6. There is evidence that genes closely associated with the MHC region are also operative in psoriasis and PsA. These include the MICA gene, S gene (corneodesmosin), and cytokine genes such as TNF-α. Psoriasis has been found to have an association with the S gene, which is expressed specifically during keratinocyte differentiation, making it a strong functional candidate for psoriasis and/or PsA (Tazi Ahnini *et al*, 1999). The A9 allele of MICA, a membrane protein similar to the MHC class I molecule, is associated with PsA independent of psoriasis (Gonzalez *et al*, 1999). Two polymorphisms in the TNF-α promoter region are also reported to be more frequent in PsA (Hohler *et al*, 1997; Al-Heresh *et al*, 1997) and certain IL-1 genotypes have been associated with psoriasis (Patel *et al*, 2001).

This study examined whether the possible differences in cytokine gene expression caused by the seven polymorphisms investigated might have an important effect *in vivo* on the level of inflammation that occurs in psoriasis and PsA. Given the observations that pro-inflammatory cytokines are increased in serum, synovium, and synovial fluid, it was hypothesised that functional polymorphism status in pro-inflammatory and anti-inflammatory genes could determine disease susceptibility and severity, as assessed by radiological erosion, in PsA. We therefore studied the gene frequencies of several cytokine gene polymorphisms in a group of patients with PsA as compared to a large control population.

5.5.2 Subjects and Methods

Controls

Blood samples were collected from 389 blood donors (Section 5.2.1).

Psoriatic Arthritis Patients

Blood samples were prospectively collected by Dr David Kane from patients with PsA

(n=147; males=74, females=73) attending at the Department of Rheumatology, St Vincent's University Hospital, Dublin, Ireland. This study was approved by the St Vincent's University Hospital ethics committee. The diagnosis of PsA was made by a consultant rheumatologist according to the criteria of Moll and Wright (Moll and Wright, 1973b). Clinical examination of all joints was carefully performed recording the pattern of peripheral and axial joint disease in addition to the presence of skin and nail disease. Patient function was categorised according to the Steinbrocker criteria for rheumatoid arthritis (Steinbrocker *et al*, 1949). PsA was classified according to the pattern of peripheral joint disease as oligoarticular (<5 joints ever involved) or polyarticular (≥5 joints involved). Spinal involvement was defined as a history of inflammatory back pain or radiological evidence of sacroileitis or spondylitis. Psoriasis was classified as type 1 psoriasis (onset at age <40 years) or type 2 (onset at age of ≥40 years).

Plain radiographs of the hands and feet were obtained and radiographs were evaluated by two trained observers in agreement. The distal interphalangeal (DIP), proximal interphalangeal (PIP), metacarpophalangeal (MCP) and carpal joints were examined in the hand. The PIP and metatarsophalangeal (MTP) joints were examined in the feet. The presence of erosions or periostitis was recorded. In patients with early onset PsA, sequential radiographs were analysed in chronological order of dates with the observers blinded to the patient identity and the total number of erosions in the hands and feet was recorded.

Psoriatic Arthritis Samples

DNA was isolated from the PsA blood samples (Section 5.2.2), and PCR and RFLP analysis was performed in order to genotype samples for seven cytokine gene polymorphisms (Sections 5.2.3-5.2.5).

5.5.3 Results

Patients with Psoriatic Arthritis

The mean ± SD age of PsA patients at arthritis onset was 35.2 ± 13.4 years (median 34 years, range 9-73 years). PsA was classified as oligoarticular in 43 patients (29%) and polyarticular in 104 patients (71%); 42 patients (29%) had spinal involvement. The mean ± SD age of PsA patients at psoriasis onset was 28.6 ± 14.2 years (median 26 years, range 1-

72 years). One hundred fifteen patients (78%) had type 1 psoriasis and 30 patients (20%) had type 2 psoriasis; in 2 patients the age of onset of psoriasis could not be determined.

Radiographic Assessment

Plain radiographs of the hands and feet were obtained in 113 patients. The median duration of disease at the time of radiological assessment was 48 months (range 1-373 months). Sixty three patients had erosions of the hands and feet, and 48 had periostitis of the hands and feet. Sequential radiographs were obtained in 51 of 61 patients who presented with a disease duration of <2 years. Of these 51 patients, 18 had increased numbers of erosions in the hands and feet and were termed "progressors", while 33 did not have increased numbers of joint erosions and were termed "non-progressors".

Cytokine Gene Polymorphisms: Susceptibility to PsA

There was no significant difference in genotype frequencies between the controls and the population of PsA patients for any polymorphism (Table 5.9).

Cytokine Gene Polymorphisms: Clinical Phenotype of PsA

No association was seen with Steinbrocker functional class, PsA disease type (polyarticular or oligoarticular), presence of disease affecting the spine, presence of periostitis, age at PsA onset, or psoriasis disease type (type 1 or 2).

The presence of joint erosions was very significantly associated with the TNF-α -308 and TNFB polymorphisms ($p<0.0001$ and $p=0.0017$ respectively) (Table 5.10). The TNF-α -308 A and the TNFB1 alleles were both significantly more frequent in the group of patients with erosions than in the group with no erosions. A similar trend was observed when looking at disease progression (Table 5.10), with a higher frequencies of the TNF-α -308 A and TNFB B1 alleles in the progressors than in non-progressors.

The TNFB1B1 genotype was associated with the highest mean age at psoriasis onset (35.3 years) and the B2B2 genotype with the lowest age at onset (22.8 years) ($p=0.0003$) (Figure 5.3). For TNF-α -308 genotypes, heterozygosity was associated with the highest mean age at onset (33.3 years), the AA genotype with the lowest mean age at onset (23.8 years), whilst the GG genotype was associated with a mean age at psoriasis onset of 26.1 years ($p=0.0081$) (Figure 5.3). Similar trends were seen for age at PsA onset, although these did not reach statistical significance (TNFB $p=0.076$; TNF-α -308 $p=0.14$).

Table 5.9 Raw data and genotype frequencies (in brackets) of seven polymorphisms in a control population and a PsA patient population.

Polymorphism	Genotype	Control Group n=389	PsA n=147
IL-6	GG	123 (32)	51 (35)
	GC	198 (51)	77 (52)
	CC	68 (17)	19 (13)
TNFB	B1B1	52 (13)	24 (16)
	B1B2	205 (53)	68 (46)
	B2B2	132 (34)	55 (38)
TNF-α -308	GG	233 (60)	85 (58)
	GA	140 (36)	55 (37)
	AA	16 (4)	7 (5)
IL-10 -1082	GG	123 (32)	51 (35)
	GA	180 (46)	53 (36)
	AA	86 (22)	43 (29)
IL-10 -592	CC	235 (60)	98 (67)
	CA	139 (36)	44 (30)
	AA	15 (4)	5 (3)
IL-1β +3953	CC	240 (62)	89 (60)
	CT	125 (32)	51 (34)
	TT	24 (6)	7 (5)
IL-1RN	A1A1	183 (47)	71 (48)
	A1A2	159 (41)	56 (38)
	A1A4	0 (0)	1 (1)
	A2A2	31 (8)	11 (8)
	A1A3	15 (4)	6 (4)
	A2A3	1 (0.2)	2 (1)

n = number of individuals.

Table 5.10 Genotype and allele counts (raw data, with percentages in brackets) of the TNF-α -308 and TNFB polymorphisms in psoriatic arthritis patients classified as presenting with or without erosions (n=63 and 50 respectively), and as disease progressors or non-progressors (n=18 and 33 respectively). χ^2 p values are shown for comparisons of genotypes or alleles between erosive and non-erosive groups and between progressor and non-progressor groups.

Polymorphism, genotype or allele	Erosive	Non-Erosive	p value	Progressor	Non-Progressor	p value
TNF-α -308						
AA	3 (5)	4 (8)	<0.0001	1 (6)	3 (9)	0.0078
GA	39 (62)	9 (18)		13 (72)	9 (27)	
GG	21 (33)	37 (74)		4 (22)	21 (64)	
A	45 (36)	17 (17)	0.0017	15 (42)	15 (23)	0.0448
G	81 (64)	83 (83)		21 (58)	51 (77)	
TNFB						
11	12 (19)	8 (16)	0.0017	2 (11)	7 (21)	0.0486
12	38 (60)	16 (32)		13 (72)	12 (36)	
22	13 (21)	26 (52)		3 (17)	14 (43)	
1	62 (49)	32 (32)	0.0091	17 (47)	26 (39)	0.4442
2	64 (51)	68 (68)		19 (53)	40 (61)	

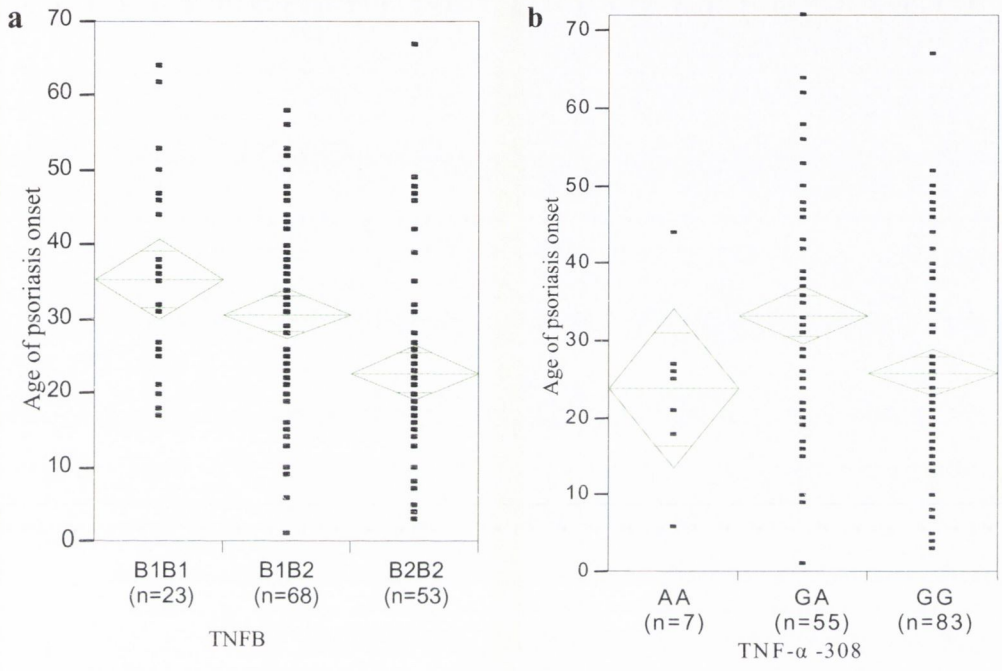

Figure 5.3 Age at psoriasis onset by TNFB and TNF-α -308 genotype: **a)** for TNFB genotypes, mean ages at onset were 35.3 years for B1B1, 30.8 years for B1B2, and 22.8 years for B2B2 (p=0.0003); **b)** for TNF-α -308 genotypes, mean ages at onset were 23.8 years for AA, 33.3 years for GA, and 26.1 years for GG (p=0.0081).

5.5.4 Discussion

Synovial inflammation in PsA is characterised by a similar degree of lymphocyte infiltration as is observed in RA, but there is less macrophage infiltration and lining layer hyperplasia with increased vascularity (Veale *et al*, 1993). In RA, it is now established that the balance of Th1 and Th2 cytokines is central to the processes of synovial inflammation and specific inhibition of the pro-inflammatory cytokines IL-1 and TNF-α produces clinical improvement (Bresnihan *et al*, 1998; Maini *et al*, 1999). The pattern of cytokine production in PsA is not fully elucidated, although studies of serum, synovial fluid, and synovium confirm the presence of increased amounts of both Th1 and Th2 cytokines (Elkayam *et al*, 2000; Partsch *et al*, 1997; Partsch *et al*, 1998a; Partsch *et al*, 1998b), the balance of which determines the features of synovial inflammation. The expression of many Th1 and Th2 cytokines is thought to be influenced by functional polymorphisms in their gene loci, and these polymorphisms may thus contribute to susceptibility or severity of inflammatory diseases. This study genotyped 147 PsA patients and 389 healthy controls for seven polymorphisms in cytokine genes, all of which are known to be expressed in PsA joints.

In this study, the frequencies of the cytokine gene polymorphisms were not found to differ significantly between the PsA patient group and the control group. This indicates that no allele or genotype of these polymorphisms predisposes to the development of PsA. However, it is possible that haplotypes of polymorphisms may be more biologically important than single polymorphism analysis, and further investigation in this area would be of interest.

Three studies have already examined TNF polymorphisms in smaller PsA and control groups and have reported an association of PsA with the TNF-α –238 polymorphism, but not with the –308 polymorphism (Hohler *et al*, 1997; Al-Heresh *et al*, 2002; Hohler *et al*, 2002). Combined analysis of the patients from 2 studies found a significant association of the TNF-α -308 G allele with PsA (Hohler *et al*, 2002), though it should be noted that up to 25% of patients had a predominant spondylitis pattern (inflammation of the vertebrae). It is of interest that the same group found a protective effect of the TNF-α –308A allele on the development of spondylitis in PsA, and thus the increase in the frequency of the G allele may reflect the presence of a significant number of patients with spondylitis. The

current study found no association of PsA with the TNF-α –308 polymorphism when compared with the control population, but, of note, none of the patients had a predominant spondylitis pattern of disease.

IL-10 is a Th2 anti-inflammatory cytokine that suppresses TNF production in monocytes. IL-10 is present in 15% of PsA synovial fluids and IL-10 levels in PsA synovial fluid are lower than those seen in RA (Partsch *et al*, 1998a) with similar levels in PsA and RA synovium (Danning *et al*, 2000). Administration of recombinant IL-10 to patients with PsA in order to restore the Th1/Th2 balance, produced a modest improvement in psoriasis but disappointingly no clinical benefit was obtained in arthritis activity (McInnes *et al*, 2001). The IL-10 –1082 G allele has been associated with PsA in one study of German patients (Hohler *et al*, 2001) but not in another study of English patients (Al-Heresh *et al*, 2002). We found a similar frequency of IL-10 –1082G allele in the PsA group as that of the German group (53% to 54%). However our control group had a higher G allele frequency than that of the German control group (55% to 41%) thus explaining the discrepancy between the studies. We found no evidence that either IL-10 polymorphism influenced PsA susceptibility or phenotype.

PsA runs a variable course, from being a mild, non-destructive disease to being a severe rapidly progressive, erosive arthropathy (Gladman and Brockbank, 2000). Identification and treatment of those patients at risk of progressive, erosive PsA is a significant challenge for the rheumatologist. A few clinical markers of poor prognosis PsA have been identified. These include an early age of onset of PsA, female gender, and the presence of polyarthritis (Gladman and Farewell, 1999). Despite the central role played by inflammatory cytokines in PsA, no study has addressed the role of cytokine production or cytokine gene polymorphisms in disease severity in PsA.

In this study, the presence of erosions of the hands and feet in PsA was found to be very significantly associated with a higher frequencies of the TNF-α -308 A allele and the TNFB1 allele. The analysis of disease progression can be problematic when using radiographs obtained at variable disease stages, since the fact that different rates of progression may occur at different stages of disease is not taken into account. To address this problem, we looked at a more homogeneous subgroup of patients presenting with early PsA (duration <2 years), and prospectively followed disease progression by obtaining sequential radiographs for a median period of two years. In this sub-group, the progression

of joint erosions in the hands and feet was also associated with the TNF-α -308 A and TNFB1 alleles. The -308 A allele is associated with high levels of TNF-α, suggesting that a genetic predisposition to produce higher levels of TNF-α is an important factor in whether a patient presents with, and subsequently develops, erosions. Significantly increased levels of TNF-α are present in PsA synovial fluid (Partsch *et al*, 1997; Ritchlin *et al*, 1998) and inhibition of TNF-α using a TNF receptor fusion protein markedly improves skin and joint inflammation in PsA (Mease *et al*, 2000). These data suggest that anti-TNF therapy in PsA may also retard joint erosion and should be considered in patients with progressive erosive PsA.

There have been several reports of the functional effects of the TNFB polymorphism; however, the results are conflicting, and the effect of this polymorphism is not yet clear. The B1B1 genotype has been linked with higher levels of TNF-β (lymphotoxin-α) (Messer *et al*, 1991; Lee *et al*, 1997), and therefore the presence of erosions in PsA may be partially caused by higher levels of TNF-β in the affected joint. However, the two TNF genes are located in the same area of the genome, and it is thus possible that any association of the TNFB polymorphism with disease could be due to linkage with the TNF-α -308 polymorphism or to linkage of both polymorphisms with another disease marker located nearby, since there is a strong association between PsA and certain HLA haplotypes (Woodrow and Ilchysyn, 1985), the genes of which are located along with the TNF locus in the MHC region, and there is evidence that genes closely associated with the MHC region are operative in psoriasis and PsA.

Serum and synovial fluid levels of both IL-1Ra and IL-6 correlate with the number of painful and swollen joints, and the level of IL-1β correlates with the extension of radiographic erosion in PsA (Elkayam *et al*, 2000; Spadaro *et al*, 1996; Nordahl *et al*, 1998). TNF-α stimulates the release of other cytokines including IL-1 and IL-6. Interestingly, we did not find an association of the IL-1β, IL-1RN, or IL-6 gene polymorphisms with disease susceptibility, phenotype, or severity in PsA. This may reflect a more central role of TNF-α in the hierarchy of inflammation in PsA. The expression of TNF-α in inflammatory arthritis is in turn regulated by other cytokines, such as IL-15 and IL-18 (McInnes *et al*, 2000; McInnes and Liew, 1998), and thus further investigation of the genetic regulation of these cytokines is warranted.

Age of onset of psoriasis was associated with the TNF-α -308 and TNFB polymorphisms. However, the results are not as conclusive as those obtained for joint destruction and progression of erosions in PsA. The earliest mean age at onset was found to be associated with the TNF-α -308 AA genotype, a producer of high levels of TNF-α, but the GG genotype was also associated with a low mean age at onset, whereas heterozygotes were found to have the highest mean age at psoriasis onset. The earliest mean age at onset was associated with the TNFB2B2 genotype, which has been linked with high levels of TNF-α (Pociot *et al*, 1991; Pociot *et al*, 1993), although these protein level findings do not correlate with the cytokine protein findings in this study (section 5.2).

The results of this study indicate that TNF-α plays an important role in both PsA and psoriasis. This is the first study to demonstrate that polymorphisms that affect TNF plasma levels are associated with the degree of joint destruction in patients with PsA. Until the functional effect of the TNFB polymorphism is determined, it will not be possible to fully elucidate the role of this polymorphism in PsA; however, it is clear that the high TNF-α-producing A allele of the TNF-α -308 polymorphism is associated with a more destructive arthropathy in PsA. These findings are potentially important for the application and development of therapeutic strategies, such as those employing anti-cytokine antibodies and anti-inflammatory cytokines. A TNF-α antagonist has already shown promise in the treatment of both PsA and psoriasis (Mease *et al*, 2000), and IL-10 administration ameliorates psoriasis (McInnes *et al*, 2001). Genotyping PsA or psoriasis patients early in the disease may help identify those with a poor prognosis and thus lead to a more aggressive treatment of their disease, possibly through the use of anti-cytokine agents.

5.6 INFLAMMATORY BOWEL DISEASE

5.6.1 Introduction

Inflammatory bowel disease (IBD), a significant health problem in the developed world, comprises two chronic relapsing and remitting inflammatory disorders of the gastrointestinal tract, ulcerative colitis (UC) and Crohn's disease (CD) (Kumar and Clark). UC affects only the rectum and colon. In contrast, CD can affect any part of the gastrointestinal tract with inflammation occurring in all bowel layers. The general symptoms of UC are malaise, lethargy, and anorexia accompanied by diarrhoea with blood and mucus, lower abdominal discomfort, and fever (Kumar and Clark), and in severe cases, death can occur as a result of malnutrition, dehydration, and anaemia. The symptoms of CD tend to be more varied, depending on its site in the digestive tract, and can occur in a mild, moderate or severe form characterized by cycles of remission and active disease. These can include some or all of the symptoms of UC (Greenberg *et al*, 1996; Munkholm *et al*, 1995; Kumar and Clark), although extra-intestinal manifestations are also common in CD, such as inflammation of the eye, skin, joints, and mouth, arterial and venous thrombosis, chronic hepatitis, and other complications involving the liver, biliary tract, and kidneys (Kumar and Clark). There is considerable overlap in the clinical features of both UC and CD, and in 10% of patients presenting with IBD it is not possible to determine whether a person is suffering from CD or UC. Both forms of IBD are more common in the developed world than the developing world. The incidence of UC is slightly greater than CD (approximately 10 per 100 000 compared with 7 per 100 000), and women are more likely to be affected. Both disorders exhibit a bimodal age distribution with a major peak at 20-40 years of age, and a minor one at 60-80 years (Kumar and Clark).

Little is known about the underlying immunopathogenesis of IBD, and much evidence for increased or decreased cytokine levels in the disease is conflicting. However, both UC and CD are generally thought to be characterised by an increase in activated macrophages and T lymphocyte produced pro-inflammatory cytokine, chemokine, and adhesion molecule expression, and an inability to adequately down-regulate immune activation. IBD is characterised by a predominantly T helper (Th) 1 type profile, involving the up-regulation of cytokines such as IL-6, IL-12, IFN-γ, and TNF-α (Gross *et al*, 1992; Stevens *et al*, 1992; Shanahan, 2002). TNF antibody therapy has be found to improve both CD and UC (van

Dullemen *et al*, 1995; Targan *et al*, 1997; Evans *et al*, 1997) indicating that this cytokine plays a pivotal role in IBD. IL-1β is also implicated in IBD, with high mRNA levels being reported in active UC specimens compared to normal specimens (Stevens *et al*, 1992). IL-1β is known to stimulate inflammatory eicosanoids, and also to induce IL-8 production, a cytokine with neutrophil-chemoattractant and neutrophil-stimulating properties, and thus important in initiation and perpetuation of the inflammatory cascade. Elevated levels of IL-8 have been detected in inflamed intestinal segments from IBD patients (Nielsen *et al*, 1997; Ina *et al*, 1997). Blocking IL-1 action with IL-1Ra in animals with immune-complex-mediated colitis reduces the severity of inflammation and lowers eicosanoid concentrations in the bowel, probably by decreasing IL-1β levels which then lowers IL-8 levels in the inflamed bowel (Cominelli *et al*, 1992; Thomas *et al*, 1991). Reduced levels of IL-10, a Th2 cytokine, is also potentially important in the pathology of IBD. Encouraging results have been obtained for IL-10 treatment in IBD, administered topically in UC and systemically in patients with chronic active CD (van Deventer *et al*, 1997; Schreiber *et al*, 1995). IL-10 knock-out mice consistently develop CD-like disease (Kuhn *et al*, 1993), however, if treated with IL-10 these mice are protected from developing gut inflammation. Macrophage and T lymphocyte activation with granuloma formation is a hallmark of the pathology of CD, and a function of IL-10 is down-regulation of macrophage activation and suppression of antigenic-specific T-cell activation. Additionally, a non-pathogenic bacterial strain capable of localised delivery of a therapeutic dose of IL-10 was found to prevent onset of colitis in IL-10 (-/-) mice and reduce colitis in dextran sulphate treated mice (Steidler *et al*, 2000) - a strategy that may lead to a similar treatment for IBD in humans as an alternative to systemic treatment.

A genetic background resulting in up-regulated inflammation leading to IBD has been suggested by a number of studies including Tysk *et al* (Tysk *et al*, 1988), who estimated the heritability of CD (1.0, 95% CI 0.34 – 1.0) and UC (0.53, 95% CI 0.24 – 1.0) using the concordance rate in twin pairs. Using 'age specific incidence data' to calculate the age-corrected empiric risk estimates, the risk of developing IBD has been determined to be 8.9% for offspring if either parent suffers IBD, 8.8% for siblings of a patient, and 3.5% for parents of an afflicted child (Roth *et al*, 1989).

The aim of this study was to compare the frequency of seven cytokine polymorphisms in IBD patients and controls. We also aimed to compare genotype frequencies in UC and CD patients, in order to highlight possible differences between these two IBD disease groups.

5.6.2 Subjects and Methods

Controls

Blood samples were collected from 389 blood donors (Section 5.2.1).

IBD Patients

Blood samples were collected prospectively by Dr Nasir Mahmud, Department of Gasteroenterology, St James' Hospital, Dublin, from 172 patients with established IBD (female, n=102; male, n=70; mean age of disease onset 30.8 years, median age of disease onset 29.5 years, range of disease onset 4-69 years). The study was approved by the hospital ethical committee and consent obtained from all participating subjects. The disease group consisted of 64 patients with Crohn's disease (females, n=46; males, n=18; mean age of disease onset 30 years, median age of disease onset 29 years, range of disease onset 4-69 years), and 108 patients with ulcerative colitis (females, n=56; males, n=52; mean age of disease onset 31.5 years, median age of disease onset 30 years, range of disease onset 12-61 years).

IBD Samples

DNA was isolated from the IBD blood samples (Section 5.2.2), and PCR and RFLP analysis was performed in order to genotype samples for seven cytokine gene polymorphisms (Sections 5.2.3-5.2.5).

5.6.3 Results

IBD and Controls

Genotype and allele frequencies for all seven polymorphisms in the control group, IBD group, UC group, and CD group are shown in Table 5.11. No association was found between any of the seven polymorphisms in this study and the occurrence of IBD, excepting the IL-1β polymorphism where the CC genotype was at a higher frequency in the disease population compared to the control population (72% vs 61%, p=0.046).

Ulcerative Colitis, Crohn's Disease, and Controls

The IBD study group can be divided into two groups – patients suffering from ulcerative colitis (UC) and those suffering from Crohn's disease (CD). A significant difference was

Table 5.11 Genotype counts (with percentages in brackets) and allele frequencies of seven functional polymorphisms in a control population and an inflammatory bowel disease patient population. Subdivisions of the IBD population into UC and CD groups are also shown.

Polymor-phism	Genotype	Control n=389	IBD n=172	UC n=108	CD n=64	Allele	Control	IBD	UC	CD
IL-6 -174	GG	123 (32)	57 (33)	43 (40)	14 (22)	G	0.57	0.58	0.61	0.54
	GC	198 (51)	85 (49)	44 (41)	41 (64)	C	0.43	0.42	0.39	0.46
	CC	68 (17)	30 (18)	21 (19)	9 (14)					
TNFB	B1B1	52 (13)	25 (15)	18 (17)	7 (11)	B1	0.40	0.39	0.38	0.41
	B1B2	205 (53)	86 (50)	47 (43)	39 (61)	B2	0.60	0.61	0.62	0.59
	B2B2	132 (34)	61 (35)	43 (40)	18 (28)					
TNF-α -308	GG	233 (60)	115 (67)	78 (72)	37 (58)	G	0.78	0.81	0.83	0.78
	GA	140 (36)	49 (28)	23 (21)	26 (41)	A	0.22	0.19	0.17	0.22
	AA	16 (4)	8 (5)	7 (7)	1 (1)					
IL-10 -1082	GG	123 (32)	62 (36)	37 (34)	24 (38)	G	0.55	0.58	0.56	0.60
	GA	180 (46)	76 (44)	48 (45)	29 (45)	A	0.45	0.42	0.44	0.40
	AA	86 (22)	34 (20)	23 (21)	11 (17)					
IL-10 -592	CC	235 (60)	117 (68)	72 (67)	44 (69)	C	0.78	0.82	0.81	0.84
	CA	139 (36)	49 (29)	31 (29)	19 (30)	A	0.22	0.18	0.19	0.16
	AA	15 (4)	6 (3)	5 (4)	1 (1)					
IL-1β +3953	CC	240 (62)	124 (72)	78 (72)	46 (72)	C	0.78	0.83	0.83	0.83
	CT	125 (32)	38 (22)	24 (22)	14 (22)	T	0.22	0.17	0.17	0.17
	TT	24 (6)	10 (6)	6 (6)	4 (6)					
IL-1RN	A1A1	183 (47)	74 (43)	48 (44)	25 (39)	A1	0.69	0.66	0.68	0.61
VNTR	A1A2	159 (41)	70 (41)	44 (41)	26 (41)	A2	0.29	0.30	0.27	0.35
	A1A4	0 (0)	1 (0.5)	1 (1)	0 (0)	A3	0.02	0.03	0.02	0.02
	A2A2	31 (8)	17 (10)	8 (7)	10 (15)	A4	0.00	0.00	0.00	
	A1A3	15 (4)	9 (5)	6 (6)	3 (5)	A5	0.00	0.00	0.00	
	A1A5	0 (0)	1 (0.5)	1 (1)	0 (0)					
	A2A3	1 (0.2)	0 (0)	0 (0)	0 (0)					

n = number of individuals.

observed when comparing the UC study group to the control population for the TNF-α -308 polymorphism, with carriers of the –308 A allele at a lower frequency in the UC group (28%) compared to the control group (40%) (p=0.02). There was no association between any of the other polymorphisms studied and the occurrence of either form of IBD when comparing with the control group, although the IL-1RN 22 genotype was almost twice the frequency in the CD group (14%) than that observed in either the control population (8%) or the UC population (7%) (p=0.088). Our study found a significant difference in the frequency of IL-6 –174 genotypes in the UC group compared to the CD group (p=0.01). A similar association was seen for the TNF-α -308 polymorphism (p=0.0135).

Age of IBD Onset

No association was seen between age of disease onset and any of the seven polymorphisms studied, either for the IBD group as a whole or when dividing into UC and CD groups.

5.6.4 Discussion

The pathophysiological mechanisms responsible for the development of IBD have not yet been fully elucidated. The main cause of disease pathology is, as the name suggests, up-regulated inflammatory processes the consequence of which is inflammatory cell infiltration in lamina propria, and crypt abscesses; goblet cell depletion in the case of UC; and inflammation in all bowel layers in CD, with lymphoid hyperplasia and an increased presence of chronic inflammatory cells (Kumar and Clark). Although infectious or other external agents are probable contributors to IBD pathogenesis, or might trigger disease onset, and the immune system clearly mediates tissue damage in the disease, it is clear from available data that genetic factors play a role in determining the susceptibility of a given individual to IBD. Since IBD is characterised by a failure to confine the usual self-limited gut inflammatory response, genes involved in determining the level of the immune response in the inflammatory pathway might be risk factors in the disease. We therefore examined the potential association of a group of polymorphisms in cytokine genes with the occurrence of IBD, or the UC and CD patient subsets.

When comparing the complete IBD population with controls, a significant variation in genotype frequency of the IL-1β promoter polymorphism was found. Higher levels of the pro-inflammatory cytokine IL-1β would be expected to increase the likelihood of

developing IBD, since higher levels of such cytokines occur in this disease. This study detected higher levels of IL-1β CC in IBD patients, a genotype associated with low IL-1β levels (Pociot *et al*, 1992), although the association of this polymorphism with variable IL-1β levels has still to be finally elucidated (see Section 5.3). It is possible that the balance of high or low levels of IL-1β is important in determining whether disease develops or in determining disease phenotype, rather than an overly pro-inflammatory genotype profile. It must also be noted, however, that the association seen in this study is only just statistically significant (p=0.046), and if a correction for multiple analyses was applied, would no longer be so.

In our study population, there was a significant variation in TNF-α -308 polymorphism genotype frequency when comparing the control group and the UC group. The control group contained a greater frequency of heterozygotes, and the UC group a greater frequency of homozygotes (especially GG), and thus the UC group had a lower frequency of carriers of the TNF-α –308 A allele. Previously, Vatay *et al* found the TNF-308 A allele to be less frequent in both UC and CD patients compared to controls (Vatay *et al*, 2003), and Boumas *et al* found a lower frequency of the –308 A allele in UC patients compared to healthy controls (Bouma *et al*, 1996). However, Louis *et al* found a modest non-statistically significant reduction of –308 A allele frequency in CD patients compared to both UC patients and controls (Louis *et al*, 1996). Another study found no difference in TNF-α –308 allele frequencies between controls and patients with fistulising CD (Gonzalez *et al*, 1999) but did not look at UC patients. It therefore appears that there may be a slight association between the occurrence of UC and non-carriage of the –308 A allele. This allele is generally associated with increased serum TNF-α levels and so the significance of the association with UC, an inflammatory condition, has yet to be fully elucidated. It is possible that this polymorphism might play a role in the differences seen between subsets of patients with UC and CD.

When comparing the two IBD disease groups, it was observed that there was a higher number of IL-6 -174 and TNF-α -308 heterozygotes in the CD group compared to the UC group for both polymorphisms. TNFB genotypes also differed between UC and CD groups, but did not reach statistical significance, and this difference was probably caused by linkage to the TNF-α –308 polymorphism. The explanation for why certain patient groups should contain a higher number of heterozygotes or homozygotes is as yet unclear. It is possible that these genotypes serve merely as genetic markers for other genes, but does

highlight a difference between the two diseases. The associations between IBD and the TNF-α polymorphism are discussed above. The IL-6 –174 polymorphism was previously investigated in 169 CD patients, 133 UC patients and 440 healthy controls, and no significant difference in allele, genotype, or carrier frequencies between patients and controls was observed (Klein *et al*, 2001). The significance of the differences of the TNF-α and IL-6 polymorphisms between the UC and CD groups in our study is not clear, but points to a potential difference in the pathophysiology of these two diseases, and the possibility that the effect of higher or lower levels of TNF- α and IL-6 may play different roles in patients with UC and CD.

The IL-10 -1082 polymorphism has been associated with susceptibility to IBD (UC or CD) and more significantly to UC alone (Tagore *et al*, 1999), however our study found no evidence to support these results, concurring with the results of Klein *et al* who looked at both –1082 and –592 polymorphisms in 142 CD patients, 104 UC patients and 400 controls and found no differences in allele frequencies of either polymorphism between the groups studied (Klein *et al*, 2000). Allele 2 of the IL-1RN gene has been found at a greater frequency in a UC population compared to a control group (Mansfield *et al*, 1994). It is interesting to note that, although not statistically significant, the IL-1RN A2A2 genotype in our study was double the frequency in the CD group compared to both UC and control groups, although this differs to the previous study where the association was for UC not CD. However, a previous study looking at IL-1RN genotype in a large IBD population (n=529) divided into IBD subgroups and in a control group (n=289) found no differences in genotype or allele frequencies, looking in particular at IL-1RN allele 2 (Craggs *et al*, 2001) and it therefore appears likely that no real association between IBD and the IL-1RN VNTR exists. This lack of association has also been confirmed by a further study (Hacker *et al*, 1997).

In conclusion, this study provides evidence that polymorphisms in genes involved in the inflammatory response are involved in determining susceptibility and disease phenotype in IBD patients, although not to a highly significant degree. Whether the polymorphisms are directly involved in regulating cytokine production and consequently disease pathophysiology of IBD, or serve merely as markers that are in linkage disequilibrium with susceptibility genes is as yet unclear. There does not appear to be any clear-cut correlation between alleles or genotypes that are associated with higher pro-inflammatory cytokine levels and disease occurrence. However, it may be that an imbalance in levels of the

cytokines is an important factor in the development of IBD, rather than an overall pro-inflammatory profile.

5.7 CARDIAC SURGERY AND LACTIC ACIDOSIS

5.7.1 Introduction

Generalised inflammation in response to infection, or any systemic insult, may result in organ failure, which is a common cause of death from critical illness (Polk and Shields, 1977). A systemic inflammatory response syndrome (SIRS) occurs in some cases after uneventful cardiopulmonary bypass (CPB), and can be associated with death, lactic acidosis, coagulopathy, systemic arterial vasodilation, acute respiratory distress syndrome, and multiple organ failure (Cremer *et al*, 1996; Hall *et al*, 1997). As with severe infection, the release of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-8 is thought to mediate the post-operative SIRS, and the anti-inflammatory cytokine IL-10 is likely to play an important role in limiting this response (Wan *et al*, 1997a).

Lactic acidosis after cardiac surgery, a manifestation of systemic inflammation and excess proinflammatory cytokine production (Cremer *et al*, 1996), is an ominous event that is associated with excess mortality among patients in shock (Davies *et al*, 2001). Peak blood lactic acid levels are powerful predictors of outcome in patients with sepsis, and high lactic acid levels in the early stages of sepsis are predictive of subsequent multiple organ failure (Bakker *et al*, 1996). In trauma patients, failure to clear excess blood lactate within 48 hours is associated with high mortality rates (Abramson *et al*, 1993), and in paediatric cardiac surgery lactic acidosis is a powerful predictor of outcome, with greater lactic acid levels associated with adverse outcome, including excess mortality (Duke *et al*, 1997). In effect, it has been found that lactic acid levels are better predictors of outcome in septic shock than either indices of oxygen delivery and consumption or plasma cytokine levels (Bakker *et al*, 1991; Marecaux *et al*, 1996).

Lactic acidosis, which is frequently observed in critically ill patients, is therefore used as a measure of resuscitation. This usage is based on the concept that lactic acid is a product of anaerobic glycolysis, and that excess lactic acid levels may reflect inadequate blood flow into the tissues. Referred to as inadequate tissue perfusion, inadequate blood flow to the tissues results in the clinical syndrome known as shock. However, excess lactic acid levels in systemic inflammation do not necessarily represent tissue hypoxia. Studies have found that sepsis does not alter either tissue oxygen delivery or extraction, and that the accumulation of lactate during sepsis is not the result of limitations in tissue oxygenation,

but is a sequelae to an increased rate of pyruvate production (Gore *et al*, 1996). It is therefore likely that lactic acidosis in sepsis may represent a direct metabolic effect of specific cytokines, and that lactic acid levels could simply be a marker for the severity of systemic inflammation. This would account for the association between lactic acid levels in early septic shock and both organ failure and mortality in patients with sepsis. It is likely that the hypermetabolism and inhibition of pyruvate dehydrogenase seen in sepsis are mediated by TNF-α and IL-1. Lactic acid may represent a metabolic marker for TNF-α activity, as *in vitro* experiments have shown that TNF-α acts to inhibit pyruvate dehydrogenase and increase lactic acid production in liver and muscle (Vary *et al*, 1998).

Thus lactic acidosis, which can occur as part of the SIRS in the first few hours after operations with the use of CPB, is thought to be a manifestation of excess cytokine production and systemic inflammation (Wan *et al*, 1997a) rather than decreased blood flow to the internal organs (Haisjackl *et al*, 1998). The lactic acidosis seen after cardiopulmonary bypass is part of a 'low systemic vascular syndrome' which is in fact a systemic inflammatory response associated with a brief burst of cytokine production (Wan *et al*, 1997b). Therefore, as with sepsis, it is likely that the elevated lactic acid levels seen in CPB patients that ultimately have a poor outcome are indicative of more severe inflammation and higher levels of pro-inflammatory cytokines. It is possible that cytokine genomic polymorphisms may modulate cytokine production and systemic inflammation in cardiac surgical patients, and thus influence the occurrence and severity of lactic acidosis.

5.7.2 Subjects and Methods

Cardiopulmonary Bypass Patients

Blood samples were collected from 30 controls that had undergone uneventful cardiac surgery, and 21 patients that had developed lactic acidosis during or after cardiac surgery. Consenting patients, scheduled for routine cardiac surgery with normal pre-operative ventricular function and uneventful surgical procedures, were recruited over a six month period by members of the Department of Anaesthesiology, St James' Hospital, Dublin. The institutional ethics committee approved this study in March 1999. Patients with a history of hepatic disease were excluded. Patients were admitted to a dedicated cardiac surgical intensive care unit after surgery with care determined by the referring cardiac surgical service. Patient care was not modified for the purpose of the study. Cardiopulmonary

bypass was performed using an open system, primed with 1500 ml of Hartmann's solution, with heparin coated circuits, and roller pumps. Suction systems were controlled.

Patient demographic characteristics and history of myocardial infarction, hypertension, congestive heart failure, vascular surgery, and diabetes were collected. The nature of the surgical procedure, duration of CPB, minimum temperature on CPB, first and last arterial blood gases on CPB, blood flows and haemoglobin concentrations which corresponded with these blood gases, and least blood flow on CPB were documented.

A case-control study was performed. Lactic acidosis patients were classified as those having arterial lactic acid level in excess of 4 mmol/L at any time in the first 24 hours after cardiac surgery, and control patients were classified as those with lactic acid never greater than 4 mmol/L in the first 24 hours after surgery. Lactic acid levels, haemodynamics, inotropic requirement, and arterial blood gases were recorded at the end of CPB, on arrival in the intensive care, and 6, 12, and 24 hours later. The duration of post-operative mechanical ventilation and the blood loss in the first 12 hours after surgery was recorded.

Cardiopulmonary Bypass Samples

DNA was isolated from the CPB blood samples (Section 5.2.2), and PCR and RFLP analysis was performed in order to genotype samples for seven cytokine gene polymorphisms (Sections 5.2.3-5.2.5).

5.7.3 Results

Age, gender distribution, body surface area, pre-operative chronic disease states, and the nature of surgical procedure were similar in both control cardiac surgery patients and lactic acidosis patients, as was the minimum temperature on CPB. CPB time was longer in lactic acidosis patients although this difference did not reach statistical significance. In the lactic acidosis group, arterial partial pressure of carbon dioxide was greater at the end of CPB (4.74 ± 0.9 pKa versus 4.5 ± 0.7 pKa, $p=0.05$) and the duration of mechanical ventilation was higher. Otherwise, arterial blood gases, haemoglobin, and circuit flow rates at the beginning and termination of CPB were similar in the two groups, as was post-operative blood loss (Table 5.12).

Table 5.12 Patient demographics and operative details. All values are quoted as frequency for categorical values and as a mean ± standard error for continuous variables.

	Control n=30	Lactic Acidosis n=21	p-value
Age (years)	60.2 ± 1.6	61.4 ± 1.9	ns
Sex (male)	28	16	ns
Body surface area (m^2)	1.96 ± 0.03	1.88 ± 0.04	ns
Haemoglobin (g/dl)	14.1 ± 0.3	14 ± 0.4	ns
Urea (mmol/l)	7 ± 0.4	6.8 ± 0.5	ns
Creatinine (mmol/l)	106 ± 3.3	105 ± 4	ns
Albumin (g/dl)	38 ± 2.4	36 ± 2.9	ns
Hypertension	9	6	ns
Myocardial infarction	6	4	ns
Diabetes mellitus	2	2	ns
CABG	27	16	
Valve	1	4	
CABG/Valve	2	1	
CPB time (min)	95 ± 4.5	109 ± 5.7	0.057
Minimum CPN temp	31.4 ± 0.3	31.8 ± 0.4	ns
Blood loss 24 hrs (ml)	657 ± 90	733 ± 109	Ns
Postoperative ventilation time (hrs)	6.8 ± 1.3	11.5 ± 1.5	0.02

n = number of individuals; CABG = coronary artery bypass graft; CPB = cardiopulmonary bypass; ns = not significant.

Table 5.13 Lactic acid levels (mmol/l) in control and study patients after surgery. All values are mean ± standard error.

	Control n=30	Lactic Acidosis n=21	p value
End CPB	1.79 ± 0.17	2.96 ± 0.2	<0.0001
Intensive care hr 1	1.47 ± 0.19	3 ± 0.23	<0.0001
Intensive care hr 6	1.54 ± 0.29	6.76 ± 0.34	<0.0001
Intensive care hr 12	1.3 ± 0.25	3.56 ± 0.3	<0.0001
Intensive care hr 24	1.61 ± 0.19	1.79 ± 0.23	<0.05

n = number of individuals; CBP = cardiopulmonary bypass.

Lactic acid levels were significantly greater in the lactic acidosis group at all times in the first 24 hours, with the greatest difference seen 6 hours after intensive care admission (Table 5.13). MANOVA for repeat measures of lactic acid over time, with patient group as a factor, found that lactic acid levels changed significantly over time ($p<0.0001$) and found an interaction between patient group and time ($p<0.0001$), indicating that patient grouping affected the temporal change in lactic acid levels. Although the duration of CPB was longer in the study group, this duration did not correlate with lactic acid levels. Lactic acid levels at 6 hours after intensive care admission correlated with the duration of mechanical ventilation. There was no association between lactic acid levels 6 hours after intensive care admission and infusion rate of epinephrine (lactic acid level mmol/litre=3.5 + 11.7 epinephrine υg/kg/min, $p=0.4$).

Looking at the whole study group, lactic acid levels were significantly higher six hours after intensive care admission in patients homozygous for the TNFB1 allele, and significantly lower in patients homozygous for the IL-10 –1082 G allele (Table 5.14). On MANOVA, with TNFB and IL-10 -1082 genotypes as factors, and analysing repeat measurement of lactic acid at 1 and 6 hours after intensive care admission, there was significant association between TNFB1 homozygosity and lactic acid level ($p=0.03$), and between IL-10 -1082 A allele carriage and lactic acid level ($p=0.03$). Lactic acid levels changed with time ($p=0.0002$), the interaction between time and TNFB genotype was significant ($p=0.0005$), as was the interaction between the IL-10 –1082 genotype and time ($p=0.01$).

Patient grouping (control or lactic acid group) was not associated with the distribution of any individual cytokine polymorphism allele, however, all 4 patients homozygous for the TNFB1 allele and who also carried the IL-10 –1082 A allele were in the lactic acidosis group ($p=0.02$). One other patient who was homozygous for the TNFB1 allele, but who did not carry the IL-10 -1082 A allele, had normal lactic acid levels. There was no association between TNF-α -308 genotype and lactic acid levels. However, only 2 patients were homozygous for the TNF-α -308 A allele. One of these carried the IL-10 –1082 A allele and developed lactic acidosis. The other did not carry the IL-10 -1082 A allele and had normal lactic acid levels. No association was seen between the IL-1β +3953, Il-6 –174, IL-1RN VNTR and IL-10 –592 polymorphisms and lactic acid levels. There was also no relation between blood pressure, central venous pressure, post-operative blood loss, and genotype.

Table 5.14 Lactic acid levels (mmol/l) after cardiac surgery in relation to TNFB and IL-10 -1082 genotype. All values are mean ± standard error.

Genotype	TNFB1/B1	TNFB1/B2	TNFB2/B2	p value TNFB	IL-10 -1082 GG	IL-10 -1082 GA or AA	p value IL-10 -1082
	n=5	n=30	n=16		n=18	n=33	
End CPB	1.9 ± 0.5	2.1 ± 0.2	2.7 ± 0.3	ns	2.2 ± 0.3	2.3 ± 0.2	ns
Intensive care hr 1	2.2 ± 0.6	2.0 ± 0.2	2.4 ± 0.3	ns	1.8 ± 0.3	2.3 ± 0.2	ns
Intensive care hr 6	7.1 ± 1.3	2.9 ± 0.5	4.1 ± 0.7	0.01	2.5 ± 0.7	4.3 ± 0.5	0.03
Intensive care hr 12	3.1 ± 0.8	1.9 ± 0.3	2.5 ± 0.4	ns	1.8 ± 0.4	2.4 ± 0.3	ns
Intensive care hr 24	2.2 ± 0.5	1.2 ± 0.2	1.6 ± 0.3	ns	1.3 ± 0.3	1.5 ± 0.2	ns

n = number of individuals; CPB = cardiopulmonary bypass; ns = not significant.

5.7.4 Discussion

Excess lactic acid accumulation after CPB has been attributed to decreased blood flow to the internal organs, referred to as splanchnic hypoperfusion, with restoration of blood flow (reperfusion) in the initial hours following surgery, and it is conceivable that regional hypoperfusion of internal organs might occur on a frequent basis during CPB. However Haisjackl *et al* found no evidence of splanchnic hypoperfusion (Haisjackl *et al*, 1998). Indeed post-CPB blood flow to the internal organs (splanchnic perfusion) and lactic acid levels were both increased compared to pre-CPB levels, suggesting that splanchnic lactic acid production after cardiac surgery is related to systemic inflammation. Cremer *et al* (Cremer *et al*, 1996) investigated the 'low systemic vascular syndrome' after CPB and found that patients with low systemic vascular resistance had greatly increased levels of TNF-α and that this excess TNF-α was always associated with a concomitant lactic acidosis. Thus lactic acid production after CPB may be a manifestation of TNF-mediated systemic inflammation rather than hypoperfusion. The association between inflammatory cytokines and excess lactic acid production is well recognised in sepsis related organ failure. In this setting, excess production of lactic acid parallels excess inflammatory cytokine production in organs that are failing (Douzinas *et al*, 1997). Vary *et al*, in an animal model, linked increased TNF-α with inhibition of pyruvate dehydrogenase and excess lactic acid production (Vary *et al*, 1998). They observed that anti-TNF antibody reversed both the TNF-mediated pyruvate dehydrogenase inhibition and the increased lactate production seen in a rat model of sepsis.

Epinephrine and other potent beta-adrenergic agonists may cause lactic acidosis (James *et al*, 1999). The mechanism for this is unclear but one hypothesis suggests that coupling of membrane bound Na/K ATPases and anaerobic glycolytic enzymes may be responsible. Lactic acidosis has been reported with epinephrine administration after CPB (Totaro and Raper, 1997), where 6 of 18 patients who received epinephrine after CPB were found to develop lactic acidosis whereas none of 17 patients in a norepinephrine group had lactic acidosis. The study did not explain why only a third of patients developed acidosis in the epinephrine group. As our study did not include patients with lactic acidosis who did not require inotropic support, the occurrence of lactic acidosis in such patients was not investigated.

In this study, routine cardiac surgical patients displayed two distinct temporal patterns of lactic acidosis. The duration of CPB may have accounted for some of this difference, yet the occurrence of post-operative lactic acidosis and the change in lactic acid levels over time was associated with the carriage of specific TNF and IL-10 alleles. A genotype was identified which was always associated with lactic acidosis, yet not all patients with lactic acidosis had this genotype.

Many polymorphisms in the TNF-α gene have been described and, of these, the functional significance and disease association of the TNF-α -308 polymorphism is best documented. However, the TNFB1 allele occurs with greater frequency than the TNF-α -308 A allele and as a consequence it is easier to investigate in a small study such as the present one, which was too small to determine the effects of the TNF-α -308 polymorphism. The B1 allele of the TNFB polymorphism was first associated with excess TNF-β production by Messer *et al* (Messer *et al*, 1991), and this trend was seen in our study of protein levels and genotype (Section 5.3), however the functionality of this polymorphism is far from clear, as discussed in Section 5.3.1. The A allele of a polymorphism at position –1082 in the IL-10 gene promoter region is associated with lower IL-10 production (Turner *et al*, 1997). Thus a genotype associated with excess TNF-β (TNFB1B1) and a decrease in IL-10 (IL-10 –1082 A) could be characterised as pro-inflammatory. We observed that all patients with this genotype had excess lactic acid. If one also considers an additional patient homozygous for the TNF-α -308 A allele, generally associated with increased TNF-α levels, and carrying the IL-10 -1082 A allele, then the association of lactic acidosis with such a pro-inflammatory genotype is even more prominent.

It is likely that the aetiology underlying the generation of systemic inflammation after CPB is multifactorial. Surgical factors, such as prolonged CPB and temperature reduction during CPB are likely to act as a trigger to precipitate systemic inflammation. This study excluded patients with prolonged, complex, or eventful surgery, thus minimising the effects of these factors, and further study is required to determine the relative importance of genetic and surgical factors in the occurrence of lactic acidosis after cardiac surgery. Despite the small size and focus on low risk patients, this study demonstrated that a combination of cytokine genetic polymorphisms may interact to promote lactic acidosis after cardiac surgery. It is possible that patients with this pro-inflammatory genotype may develop systemic inflammation and lactic acidosis after a lower threshold stimulus. However, only 20% of patients in the study group had the identified genotype. Therefore

this pro-inflammatory genotype was sufficient but not necessary to initiate systemic inflammation and further investigation is required to determine whether other genetic factors are associated with lactic acidosis in cardiac surgical patients. Having genetic information on an individual's inflammatory response prior to surgery may have distinct benefits. Along with the scientific interest concerning the role and interaction of the inflammatory mediators, there could be very practical clinical benefits, as those patients with a genetic predisposition to high cytokine release may benefit from anti-inflammatory mediator strategies.

5.8 STROKE

5.8.1 Introduction

Ischaemic stroke is a major complication of atherosclerotic cardiovascular disease, resulting in considerable morbidity and mortality (Ross, 1993). Traditional risk factors such as hypertension, diabetes, smoking and obesity do not fully account for individual risk (Anderson *et al*, 1991; Heller *et al*, 1984). Inflammatory reactions have been implicated in increasing the risk of both stroke (Macko *et al*, 1996) and myocardial infarction (Roivainen *et al*, 2000), and the expression levels of the pro-inflammatory cytokines IL-6, TNF-α, and IL-1β have been found to be increased following acute stroke (Kim *et al*, 1996; Liu *et al*, 1993; Liu *et al*, 1994). This is possibly linked to the loss of anti-inflammatory and anti-coagulant action caused by low levels of circulating APC that have been detected in stroke patients compared to both neurological controls and community control subjects (Macko *et al*, 1996). Lower circulating APC levels have also been associated with positive infection/inflammation status in both stroke and control groups (Macko *et al*, 1996). Inflammatory mediators such as IL-1 and TNF are known to reduce thrombomodulin from the endothelial cell surface, resulting in downregulation of the thrombomodulin-dependent protein C activating capacity; a process that most likely contributes to inflammation-mediated disseminated intravascular coagulation (Esmon *et al*, 1991; Taylor *et al*, 1987). Therefore, it appears that increased pro-inflammatory cytokine levels and inflammation may contribute to lowered APC levels and a predisposition to stroke. Given the observations that levels of the pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 are elevated in ischaemic stroke, we investigated whether polymorphisms in genes encoding cytokines play a role in the pathophysiology of ischaemic stroke.

5.8.2 Subjects and Methods

Controls

Blood samples were collected from 389 blood donors (Section 5.2.1).

Stroke Patients

Blood samples were prospectively collected by Dr Sean Pittock (Dept of Neurology, Beaumont Hospital, Dublin) from a consecutive cohort of ischaemic stroke patients (n=105; males 60%, females 40%; mean age of stroke 69 years, range 35-99 years)

admitted to Beaumont Hospital over a nine month period. The study was approved by the hospital ethical committee, and consent obtained from all participating subjects.

Stroke was defined as acute onset of neurological deficit lasting more than 24 hours or leading to death with no apparent cause other than cerebrovascular disease. The diagnosis of stroke was based on history and examination. Transfers from other hospitals and patients with acute intracerebral hemorrhage, subdural, extradural or subarachnoid hemorrhage were excluded on the basis of cranial imaging.

Patients were allocated to one of four stroke subtypes (Oxfordshire Community Stroke Project, OCSP) and level of handicap assessed by a study neurologist (SP) at three time points within 48 hours of admission and at two weeks and six months using the Oxford handicap scale also known as the modified Rankin Disability Scale, an observer-rated, global measure of handicap assessing any limitation in the patient's social role. It is rated from 0 (no symptoms) to 5 (severe handicap, totally dependent, requiring constant attention night and day) and has been shown to have good interrater reliability (Bamford *et al*, 1991; Bamford *et al*, 1989).

Stroke Samples

DNA was isolated from the stroke blood samples (Section 5.2.2), and PCR and RFLP analysis was performed in order to genotype samples for six cytokine gene polymorphisms (Sections 5.2.3-5.2.5). It was not possible to genotype stroke patients for the IL-10 –1082 polymorphism due to low levels of available DNA samples.

5.8.3 Results

Of 117 consecutive ischemic stroke patients enrolled in the study, 105 consented to give blood for genetic analysis. All patients were Caucasian. The demographic and clinical characteristics of this study population are shown in Table 5.15. The mean age of the cohort was 69 years (SD 12.6) and the male to female ratio was 1.5:1. There were no statistical differences in respect of gender for any of the clinical characteristics studied.

Age of First Incidence of Stroke – Association with Sex, Smoking, and Hypertension

The mean age of first incidence of stroke was calculated for smokers, ex-smokers (individuals who had not smoked for >1 year), and non-smokers. The mean age of first

Table 5.15 Patient demographics, pre-stroke function, risk factors, and stroke subtyping. Percentage of total stroke population is shown in brackets.

Risk factors	
Any prior cerebrovascular event	38 (36)
Previous cerebrovascular accident (CVA)	25 (24)
Previous transient ischaemic attack (TIA)	27 (26)
Smokers (at any time)	66 (63)
Hypertension	48 (46)
Previous arterial event	33 (31)
Pre-stroke function	
Oxford handicap scale (OHS) <2	82 (78)
Oxford handicap scale (OHS) ≥2	23 (22)
Stroke subtype	
Total Anterior Circulation Infarcts (TACI)	29 (28)
Lucunar Circulation Infarcts (LACI)	40 (38)
Partial Anterior Circulation Infarcts (PACI)	23 (22)
Posterior Circulation Infarct (POCI)	13 (12)
Rankin disability scale (6 months post event)	
0 No Symptoms	15 (12)
1 Minor Symptoms	16 (13)
2 Some Restriction	24 (20)
3 Significant Restriction	14 (11)
4 Severe Handicap	10 (8)
5 Severe Handicap – Totally Dependant	5 (4)
6 Dead	20 (16)

stroke was lower for smokers (65.7 years; range 35-86; median 59, n=38), when compared to non-smokers or ex-smokers (71 years, range 43-99; median 70, n=66) (p=0.036). When looking at variation in age of first incidence in relation to patient sex, the mean age for a first event for females was 72 (n=42) compared to 67.6 years for males (n=63) (p=0.075). Smoking had a greater effect on the age of first event in females (smokers, mean=65.3; non-smokers/ex-smokers, mean=74.6; p=0.042) compared to males (smokers, mean=66; non-smokers/ex-smokers, mean=68.6; p=0.38). Finally, those patients with hypertension had an earlier mean first event (67.6 years) compared to those with no hypertension (70.4 years), although this was not a statistically significant difference (p=0.26).

Genotype and Allele Frequencies of Six Cytokine Gene Polymorphisms

Genotype frequencies for all six polymorphisms in the control group, stroke population, and subgroups of the stroke population (previous arterial event, smoker, ex-smoker, non-smoker, and those suffering from hypertension) are listed in Table 5.16. No significant variation between the control and stoke populations was identified. Moreover, there was no significant difference found for any of these polymorphisms and stroke subtypes as defined by the OCSP. The subgroups of level of handicap (Rankin Disability Scale) were too numerous and thus contained too few individuals to determine any association with genotype. The prevalence of IL-6 -174 G allele carriers was higher in the group of stroke patients with no history of hypertension compared to those with hypertension (98% vs 77%, p=0.0009 'no hypertension' vs 'hypertension'), and compared to the control group (98% vs 83%, p=0.0027 'no hypertension' vs controls). No other significant associations were observed amongst the different groups within the stroke population, although the A allele of the TNF-α -308 polymorphism was increased in the group of stroke patients who have suffered a previous arterial event (p=0.07).

5.8.4 Discussion

Stroke is a complex disease with multiple risk factors. These include advanced age, smoking, hypertension, diabetes, coagulation disorders, low apo E levels, and abnormal cholestrol levels (Rastenyte *et al*, 1998). The increased risk of stroke in smokers is clearly demonstrated in this study, where it was found that smokers have a significantly earlier age of incidence of stroke compared to non- or ex-smokers. This was also the case for hypertension, although the influence of this risk factor was not found to be as great as the influence of smoking. From our results, it also appears that the sex of an individual can

Table 5.16 Genotype counts (with percentages in brackets) for cytokine polymorphisms in a control population and a stroke patient population. The stroke population is subdivided into those with a previous event (stroke or myocardial infarction), smoker, ex-smoker or non-smoker, and individuals with hypertension or no hypertension.

Polymorphism	Genotype	Control Group n=389	Stroke n=105	Previous Arterial Event n=33	Smoker n=38	Ex-Smoker/ Non-Smoker n=66	Hyperten-sion n=48	No Hypertension n=55
IL-6 -174	GG	123 (32)	33 (32)	13 (39)	13 (34)	20 (30)	15 (31)	18 (33)
	GC	198 (51)	60 (57)	14 (43)	21 (55)	38 (58)	22 (46)	36 (65)
	CC	68 (17)	12 (11)	6 (18)	4 (11)	8 (12)	11 (23)[a]	1 (2)[a]
TNFB	B1B1	52 (13)	16(15)	6 (18)	6(16)	10 (15)	5 (11)	11 (20)
	B1B2	205 (53)	53 (51)	14 (43)	20 (53)	32 (49)	28 (58)	24 (44)
	B2B2	132 (34)	36 (34)	13 (39)	12 (31)	24 (36)	15 (31)	20 (36)
TNF-α -308	GG	233 (60)	59 (56)	15 (45)	18 (47)	41 (62)	25 (52)	33 (60)
	GA	140 (36)	40 (38)	15 (45)	17 (45)	22 (33)	21 (44)	18 (33)
	AA	16 (4)	6 (6)	3 (10)	3 (8)	3 (5)	2 (4)	4 (7)
IL-10 -592	CC	235 (60)	65 (62)	21 (64)	22 (58)	43 (65)	27 (56)	38 (69)
	CA	139 (36)	35 (33)	11 (33)	13 (34)	21 (32)	21 (44)	12 (22)
	AA	15 (4)	5 (5)	1 (3)	3 (8)	2 (3)	0 (0)	5 (9)
IL-1β +3953	CC	240 (62)	66 (63)	24 (73)	22(58)	43 (65)	32 (67)	32 (58)
	CT	125 (32)	35 (33)	7 (21)	16(42)	19 (29)	15 (31)	20 (36)
	TT	24 (6)	4 (4)	2 (6)	0(0)	4 (6)	1 (2)	3 (6)
IL-1RN	A1A1	183 (47)	49 (47)	14 (43)	17(45)	32 (48)	22 (46)	27 (49)
VNTR	A1A2	159 (41)	41 (39)	15 (45)	16(42)	25 (38)	19 (40)	21 (38)
	A1A3	15 (4)	6 (6)	2 (6)	4(10)	1 (2)	2 (4)	3 (6)
	A2A2	31 (8)	9 (9)	2 (6)	1(3)	8 (12)	5 (10)	4 (7)

[a] χ^2 p value for hypertension vs no hypertension = 0.0084.
n = number of individuals.

have a bearing on age of first stroke, where males generally suffer from earlier stroke compared to females. However, smoking seems to be a greater risk factor for females, with a mean age of first incidence of 65.3 years for female smokers and 74.6 years for female non-/ex-smokers.

Several of the risk factors for stroke such as diabetes, hypertension, and coagulation disorders, are likely to be under genetic influence. Moreover, twin and family studies give evidence for a genetic background to stroke (Brass *et al*, 1992; Kiely *et al*, 1993), and it has been found that significantly more patients who suffer a stroke have a family history of stroke or heart disease compared to age- and sex-matched controls (Graffagnino *et al*, 1994). However, many subjects with one or more of the risk factors mentioned at the beginning of this section do not develop stroke, and some patients who suffer a stroke do not have these risk factors, indicating that other factors may be involved in the pathophysiology of stroke. With this in mind, and with an increasing amount of evidence for the involvement of pro-inflammatory reactions in the pathophysiology of ischaemic stroke (Macko *et al*, 1996; Roivainen *et al*, 2000; Kim *et al*, 1996; Liu *et al*, 1993; Liu *et al*, 1994), the prevalence of a range of polymorphisms in cytokine genes in a stroke population and in a large control population was investigated.

No substantial link between any polymorphism in this study and the occurrence of stroke could be established. Of note, however, a significant increase in the frequency of the IL-6 –174 G allele carriers in the group of ischaemic stroke patients with no hypertension was detected compared to those with hypertension and compared to controls. Therefore, the IL-6 -174 polymorphism may be associated with stroke when patients with hypertension, a known risk factor, are removed from the study group. The IL-6 –174 CC genotype is associated with low levels of IL-6 in normal controls, and has been found at a reduced frequency in young systemic-onset juvenile chronic arthritis patients, indicating a protective role in this disease, and the G allele has been associated with increased IL-6 plasma levels (Fishman *et al*, 1998). The expression levels of circulating IL-6 have been found to be increased following acute stroke (Kim *et al*, 1996), and thus the lower levels of IL-6 associated with the C allele may be protective in individuals who do not have hypertension, a stroke risk factor.

To conclude, the incidence of stroke is not significantly associated with any of the functional cytokine polymorphisms studied, although the IL-6 polymorphism may be

associated with stroke when patients with the known risk factor hypertension are removed from the study group. A larger study is needed to confirm this association. A larger patient cohort is also required to investigate further the association of inflammatory polymorphisms and stroke subtype in order to overcome the lack of statistical power when the patient population is split into smaller subtypes. Despite the evidence for the involvement of inflammation in the pathophysiology of stroke, there is little evidence as yet that inflammatory polymorphisms are a risk factor. Other genetic risk factors, such as ones in the coagulation and haemostasis systems are currently being investigated (Tatlisumak and Fisher, 1996), and it is possible that polymorphisms in this area could be of greater importance in the occurrence of stroke than polymorphisms of the inflammatory system.

CHAPTER 6

General Conclusions and Future Prospects

6.1 GENERAL CONCLUSIONS

6.1.1 Anti-Inflammatory Properties of the Protein C Pathway

APC is a naturally occurring anti-coagulant that functions by inactivating FVa and FVIIIa. APC is also known to have anti-inflammatory effects and so the PC pathway provides an important link between the coagulation, fibrinolysis, and inflammation pathways. PC/APC is one of few therapies shown to effectively reduce the morbidity and mortality levels seen in severe sepsis and septic shock. However, the mechanism by which PC/APC exerts its anti-inflammatory effects through modulation of the activated monocyte is not fully understood. Binding of PC/APC to the monocyte appears to regulate intracellular signalling and the monocytic response to inflammatory stimuli. Given the significance of the activated monocyte in the pathophysiology of severe sepsis and the recent discovery of expression of the endothelial protein C receptor (EPCR) on the monocyte cell surface, we investigated the effect of PC on the gene expression profile of the monocytic cell line THP-1.

Microarray technology was used to determine the effect of PC on the expression levels of approximately ten thousand human genes in untreated and LPS activated THP-1 cells. Results were confirmed by investigating the expression of a number of genes included in the microarray by real-time PCR and ELISA. We showed that PC effectively inhibits the induction of all LPS induced genes, most notably many chemokines and inflammatory cytokines. However, PC was not found to have a significant effect on gene expression levels in unstimulated monocytes, nor did it affect genes involved in apoptosis as previously reported in endothelial cells (Joyce *et al*, 2001; Mosnier and Griffin, 2003). This corresponded to previous research showing no effect of APC or PC on apoptosis in either neutrophils and eosinophils (Feistritzer *et al*, 2003; Sturn *et al*, 2003). These results highlighted differences between monocytes and endothelial cells with regard to the effect of APC treatment, and this underlines the different roles played by these two cell types in the pathophysiology of severe sepsis. In an effort to determine the mechanism of protein C anti-inflammatory activity, we investigated the effect of this protein on levels of

unstimulated and LPS-stimulated TLR4 and CD14, two LPS receptors on the monocyte. Despite an effect on LPS-stimulated levels of TLR4, due to the length of time for this effect to take place and due to little effect on unstimulated levels, we concluded that PC does not primarily act by downregulating levels of the LPS receptors. The observed effect was thought to be as a result of the downregulation of all LPS-induced genes by PC. The precise mechanism of action of PC on the activated monocyte is therefore still to be fully determined.

6.1.2 Genetic Predisposition to Inflammatory Disease

Inflammatory diseases are a major cause of mortality and morbidity in humans. Deficiencies in the inflammatory process can have severe consequences for a host and result in a wide range of diseases encompassing IBD, type I diabetes mellitus, Alzheimer's disease, multiple sclerosis, atherosclerosis, rheumatoid arthritis, psoriasis, systemic lupus erythematosus, neisserial or pneumococcal meningitis, mucocutaneous leishmaniasis, and systemic inflammatory response syndrome (SIRS). Such diseases are generally thought to be caused by excessive, prolonged, or dysregulated inflammation, or recognition of self tissue as foreign, although the underlying cause of dysregulted inflammation is unknown in many cases.

This study investigated several diseases associated with inflammatory pathologies and in some cases coagulopathies. These included meningococcal sepsis, psoriatic arthritis (PsA), IBD, lactic acidosis following cardiopulmonary bypass, and ischaemic stroke. These are multifactorial diseases, conveyed through complex interactions between both aquired (environmental) and genetic risk factors. However, the majority of the genetic abnormalities underlying the development of such diseases in the general population are still unknown. In order to improve the prospects of accurately predicting or determining individuals more at risk for certain inflammatory diseases, and to aid in the development of the most appropriate treatment strategies for these diseases, a more complete knowledge and understanding of the genetic basis of these diseases is vital.

Two strategies are commonly used to search for genetic risk factors in disease. One strategy involves linkage analysis of a large range of chromosomal markers to identifiy areas of the genome containing genes possibly associated with the disease under investigation. However, we utilised the candidate gene approach in our search for genetic

abnormalities responsible for increased risk or severity of the five diseases mentioned above. We investigated mutations in candidate genes involved in the inflammatory response. In many cases, published evidence had previously suggested links between the mutations studied and certain inflammatory diseases or links with increased or decreased levels of inflammatory cytokines.

In this study, assays were designed and optimised for rapid and accurate genotyping of a set of seven polymorphisms in cytokine genes - six restriction fragment length polymorphisms (RFLPs) and one variable number tandem repeat (VNTR) polymorphism - and association studies were carried out using a large control population (blood donors) and IBD, PsA, meningococcal sepsis, lactic acidosis, and stroke patient populations. A number of significant associations were found in this study. Of particular interest was the association of the IL-6 -174 and IL-10 -1082 polymorphisms with meningococcal sepsis disease severity, implying an increased risk for severe disease in individuals carrying a putative pro-inflammatory pair of genotypes. Also of interest was the highly significant association of the TNF-α -308 and TNFB mutations with the presence of erosions in psoriatic arthritis. This indicates the importance of increased TNF-α in the development of joint erosions in this disease and correlates wtith previous published findings of significantly increased levels of TNF-α in PsA synovial fluid (Partsch *et al*, 1997; Ritchlin *et al*, 1998). Additionally, inhibition of TNF-α using a TNF receptor fusion protein markedly improves skin and joint inflammation in PsA (Mease *et al*, 2000). These data suggest that anti-TNF therapy in PsA may also retard joint erosion and should be considered in patients with progressive erosive PsA.

6.2 FUTURE PROSPECTS

In order to develop the most effective therapies for the treatment of sepsis, a complete understanding of the processes involved in the disease and the mechanisms by which the host responds to these processes is of importance. Given the significant role of the monocyte in the inflammatory response and the proven effectiveness of activated protein C therapy in severe sepsis, analysis of the biological effect of APC on the human monocyte appears to be a critical objective in the effort to design increasingly effective treatment strategies for this important disease.

The natural progression from the work presented here is to further investigate the interaction between protein C and the monocyte and to elucidate fully the mechanism by which protein C attenuates the inflammatory response of the monocyte. The findings here and in other recent studies that APC/PC acts differently on monocytes and neutrophils compared to endothelial cells indicate that the overwhelming focus on the involvement of the endothelium in the inflammatory process that occurs during severe sepsis needs to be balanced with further investigation of the role of cells of the innate immune system in the disease.

APC/PC and LMWH do not appear to function by initially reducing TLR4 or CD14 levels in order to dampen the monocyte activation by LPS. Thus other possible actions must be explored. Due to the immediate effect of PC on the LPS activation of monocytes and the minimal effect of PC on the gene expression profile of untreated cells, a likely explanation for the mechanism by which PC dampens the LPS activation of monocytes is the inhibition of LPS binding to the monocyte cell surface. Thus further elucidation of the mechanism of PC binding to the EPCR on the monocyte cell surface and the possible interaction with members of the LPS activation cluster may prove beneficial. Other possible avenues of investigation in this area involve the effect of PC on IκB degradation, study of possible endocytosis of PC bound to the EPCR and identification of inhibitors of this endocytosis, elucidation of the role of PAR-1 in the monocyte, and further investigation of possible APC or PC inducible genes and intracellular signalling molecules potentially activated by binding of APC/PC to the EPCR. Of additional interest would be the use of mutants and blocking antibodies to further elicidate the roles played by APC, PC, PAR-1, EPCR, and TM, amongst others, in the inflammatory and coagulation cascades activated in severe sepsis and in response to LPS and other inflammatory stimuli by monocytes and various other cell types. For example, the use of a mutant PC which can not be activated would aid in elucidating the need for activation of PC in order to achieve the anti-inflammatory effects observed in this study.

Further gene profile experiments utilising APC instead of PC would clarify the difference, if any, between the effects of these two proteins on human monocytes. Additionally, similar experiments looking at the effect of both APC and PC on LPS-stimulated and unstimulated human primary monocytes would aid in establishing any major differences in the use of the THP-1 monocyte cell line as opposed to isolated human monocytes. Furthermore, gene profile experiments investigating the effect of LMWH on unstimulated

and LPS-stimulated monocytes would give insight into similarities or differences in the effect of these two anti-coagulant and anti-inflammatory compounds, LMWH and PC, on the activated monocyte.

In comparison to genetic risk factors such as FV Leiden and prothrombin G21210A in venous thromboembolism, the cytokine gene mutations studied here are not well-established risk factors in the development of inflammatory disease. Although many studies have been undertaken to determine association between such polymorphisms and disease, many of the results have been contradictory. It is becoming clear that even larger scale case control studies are necessary and any suggested phenotype-genotype relationships so far detected are merely indicators at most as to real associations between genotype and disease. However, the mutations chosen for study were selected following previous reports of functional effects on cytokine levels. Although initial investigations may be unrewarding in comparison to the Factor V Leiden polymorphism in VTE, for example, this selection and analysis is important for subsequent investigations into the synergistic effects of such factors in multifactorial diseases. The significant results obtained in this study are of importance in the understanding of the pathophysiology of the inflammatory diseases studied and may help to elucidate the critical cytokines involved in the diseases as well as aiding in assessing risk of more severe disease in certain individuals carrying several known risk factors. In order to progress from these initial findings, it would certainly be of interest to expand the patient groups, allowing validation of any observed associations and further exploration of the possible importance of carriage of several risk factors in the development and severity of inflammatory disease. The current findings for the functionality of the cytokine polymorphisms studied here are somewhat inconclusive and thus the proposal by Warle *et al*, of setting up a multicenter workshop in which the relationship between certain cytokine gene polymorphisms and *in vitro* cytokine production could be analysed using an identical *in vitro* cell culture system and study population (Warle *et al*, 2003), may aid in definitively determining the functionality of these mutations. Significant findings from this study can also be further investigated, for example the expansion of the PsA study to an investigation of the levels of TNF-α in the synovial fluid of the joint and the relation of these levels to TNF-α polymorphism genotype and extent of joint erosions would be of interest.

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APPENDIX A

IL-6 –174 G/C Polymorphism

IL-6 gene promoter region (G allele):

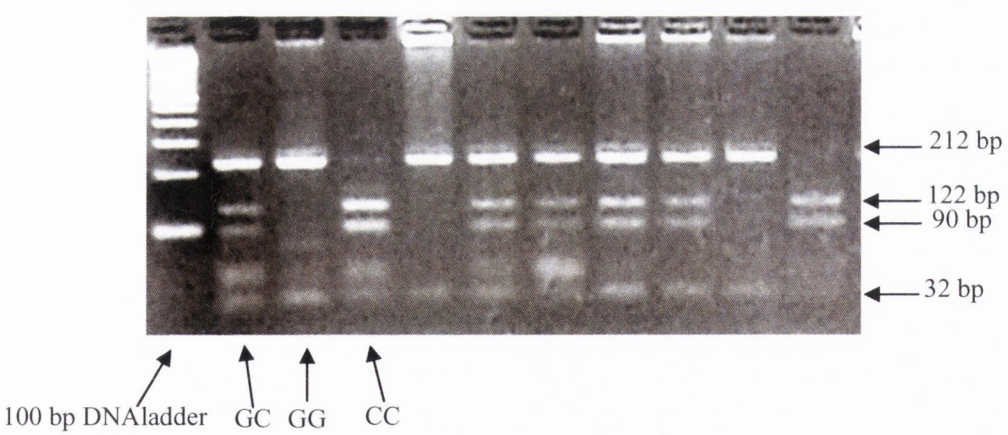
```
ttttttcaaa aaacatagct ttagcttatt ttttttctct ttgtaaaact tcgtgcatga
                                  ▼Hsp 92 II control site
cttcagcttt actctttgtc aagacatgcc aaagtgctga gtcactaata aaagaaaaaa
agaaagtaaa ggaagagtgg ttctgcttct tagcgctagc ctcaatgacg acctaagctg
cacttttccc cctagttgtg tcttgcggatg ctaaaggacg tcacattgca caatcttaat
                                  ▲Hsp 92 II restriction site (dependant C -174)
aaggtttcca atcagcccca cccgctctgg ccccaccctc accctccaac aaagatttat
```

Note: Base that changes from G to C at position –174 is boxed. Upstream and downstream primer sequences are highlighted in blue. Sequence NCBI Genbank accession number: Y00081.

Hsp 92 II cuts at 5'-CATG▼-3'

3'-GTAC▲-5'

Hsp 92 II digestion products of the IL-6 gene region containing the –174 polymorphism:



Note: DNA bands were separated by electrophoresis in 3% agarose gel containing ethidium bromide and visualised under UV light.

Wild type homozygotes (GG) produce two DNA fragments of length 32 bp and 212 bp. CC homozygotes produce three DNA fragments of 32 bp, 90bp, and 122 bp. Heterozygotes (GC) produce four DNA fragments of 32 bp, 90 bp, 122 bp, and 212 bp.

TNF-α -308 G/A Polymorphism

TNF-α gene promoter region (G allele):

tagaaggaaa cagaccacag acctggtccc caaaagaaat ggaggcaata ggttttgagg

▼ *NcoI* restriction site (dependant G -308)

gCcatgggga cggggttcag cctccagggt cctacacaca aatcagtcag tggcccagaa

gacccccctc ggaatcggag cagggaggat ggggagtgtg aggggtatcc ttgatgcttg

▼ *NcoI* control site

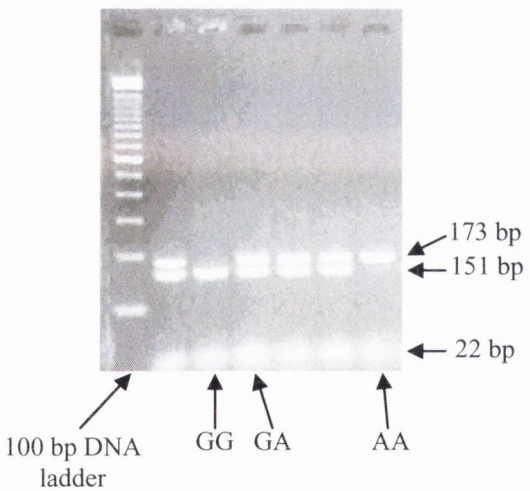
tgtgtcccca actttccaaa tccccgcccc cgcCatggag aagaaaccga gacagaaggt

Note: Base that changes from G to A at position –308 is boxed. Uppercase C denotes bases that are G in the gene sequence but C in the primer, creating Nco I restriction sites (control site and polymorphism site) in the PCR product. Upstream and downstream primer sequences are highlighted in blue/underlined. Sequence NCBI Genbank accession number: M16441.

Nco I cuts at 5'-C▼CATGG-3'

3'-GGTAC▲C-5'

Nco I digestion products of the TNF-α gene region containing the –308 polymorphism



Note: DNA bands were separated by electrophoresis in 2% agarose gel containing ethidium bromide and visualised under UV light.

Wild type homozygotes (GG) produce two DNA fragments of length 22 bp and 151 bp. AA homozygotes produce two DNA fragments of 22 bp and 173 bp. Heterozygotes (GA) produce three DNA fragments of 22 bp, 151 bp, and 173 bp.

TNF-β +252 G/A Polymorphism

TNF-β gene intron 2 region (G allele):

gcctcggtcc ctcctgcacc tgctgcctgg atccccggcc tgcctgggcc tgggccttgg

▼ *Hinf I* control site

tgggtttggt tttggtttcc ttctctgtct ctgactctcc atctgtcagt ctcattgtct
ctgtcacaca ttctctgttt ctgccatggt tcctctctgt tcccttcctg tctctctctg

▲ *Hinf I* restriction site (dependant A +252)

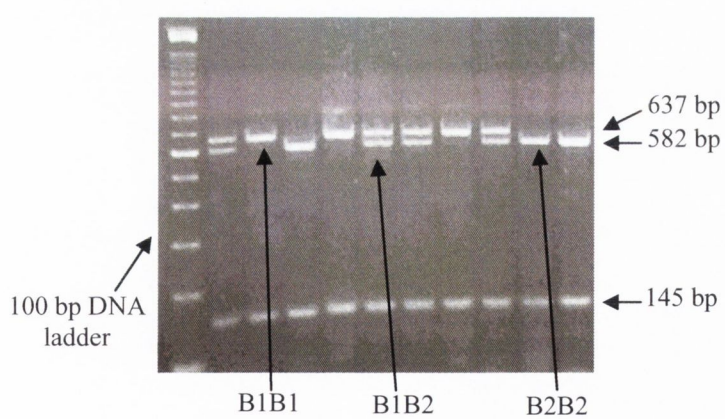
tctccctctg ctcaccttgg ggtttctctg actgcatctt gtcccttct ctgtcgatct
ctctctcggg ggtcgggggg tgctgtctcc cagggcggga ggtctgtctt ccgccgcgtg
ccccgccccg ctcactgtct ctctctctct ctctctttct ctgcaggttc tccccatgac
accacctgaa cgtctcttcc tcccaagggt gtgtggcacc accctacacc tcctccttct
ggggctgctg ctggttctgc tgcctggggc ccaggtgagg cagcaggaga atgggggctg
ctggggtggc tcagccaaac cttgagccct agagcccccc tcaactctgt tctcccctag
gggctccctg gtgttggcct cacaccttca gctgcccaga ctgcccgtca gcaccccaag
atgcatcttg cccacagcac cctcaaacct gctgctcacc tcattggtaa acatccacct
gacctcccag acatgtcccc accagctctc ctcctacccc tgcctcagga acccaagcat
ccacccctct cccccaactt cccccacgct aaaaaaaaca gagggagccc actcctatgc

Note: Base that changes from G to A at position +252 is boxed. Upstream and downstream primer sequences are highlighted in blue. Primers from (Stüber *et al*, 1996). Sequence NCBI Genbank accession number X02911.

Hinf I cuts at 5'-G▼ANTC-3'

3'- C▲TNAG-5'

Hinf I digestion products of the TNF-β gene region containing the +252 polymorphism:



Note: DNA bands were separated by electrophoresis in 2% agarose gel containing ethidium bromide and visualised under UV light.

B1B1 homozygotes (GG) produce two DNA fragments of length 637 bp and 145 bp. B2B2 homozygotes (AA) produce three DNA fragments of 55 bp, 145bp, and 582 bp. B1B2 heterozygotes (GA) produce four DNA fragments of 55 bp, 145 bp, 582 bp, and 637 bp.

IL-10 -1082 G/A Polymorphism

IL-10 gene promoter region (A allele):

ccagatatct gaagaagtcc tgatgtcact gccccggtcc ttccccaggt agagcaacac

Mnl I control sites

tcctcgccgc aacccaactg gctcccctta ccttctacac acacacacac acacacacac

Mnl I restriction sites (dependant G –1082)

acacacacac acacacacaa atccaagaca acactactaa ggcttctttg ggaaggggaa

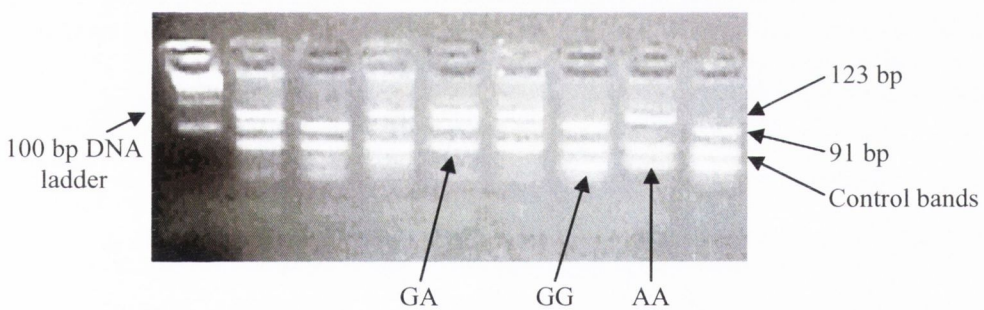
gtagggatag gtaagaggaa agtaagggac ctcctatcca gcctccatgg aatcctgact

Note: Base that changes from G to A at position –1082 is boxed. *Mnl I* cut sites are underlined and shown in red, overlapping sites in pink. Upstream and downstream primer sequences are highlighted in blue. Sequence NCBI Genbank accession number: X78437.

Mnl I cuts at $CCTC[N]_7$▼

$GGAG[N]_6$▲

Mnl I digestion products of the IL-10 gene region containing the -1082 polymorphism:



Note: DNA bands were separated by electrophoresis in 4% agarose gel containing ethidium bromide and visualised under UV light.

GG homozygotes produce six DNA fragments of length 91 bp, 45 bp, 20 bp, 17 bp, 15 bp, and 2 bp. AA homozygotes produce four DNA fragments of 123 bp, 45 bp, 20 bp, and 2 bp. Heterozygotes produce seven DNA fragments of 123 bp, 91 bp, 45 bp, 20 bp, 17 bp, 15 bp, and 2 bp.

IL-10 -592 C/A Polymorphism

IL-10 gene promoter region (C allele):

tcttttcctt gttatttcaa cttcttccac cccatctttt aaactttaga ctccagccac
agaagcttac aactaaaaga aactctaagg ccaatttaat ccaaggtttc attctatgtg
ctggagatgg tgtacagtag ggtgaggaaa ccaaattctc agttggcact ggtgtaccct

Rsa I control sites

tgtacaggtg atgtaacatc tctgtgcctc agtttgctca ctataaaata gagacggtag
gggtcatggt gagcactacc tgactagcat ataagaagct ttcagcaagt gcagactact
cttacccact tcccccaagc acagttgggg tgggggacag ctgaagaggt ggaaacatgt
gcctgagaat cctaatgaaa tcggggtaaa ggagcctgga acacatcctg tgaccccgcc

Rsa I restriction site (dependant A -592)

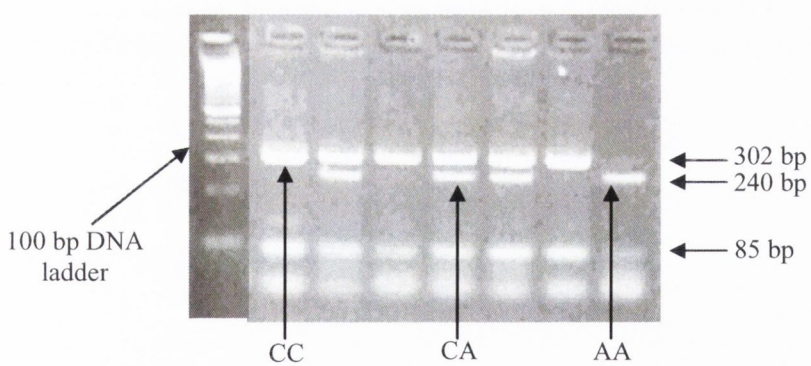
tgtcctgtag gaagccagtc tctggaaagt aaaatggaag ggctgcttgg gaactttgag
gatatttagc ccacccccctc atttttactt ggggaaacta aggcccagag acctaaggtg

Note: Base that changes from C to A at position –592 is boxed. Upstream and downstream primer sequences are highlighted in blue. Sequence NCBI Genbank accession number: X78437.

Rsa I cuts at 5'-GT▼AC-3'

3'-CA▲TG-5'

Rsa I digestion products of the IL-10 gene region containing the -592 polymorphism:



Note: DNA bands were separated by electrophoresis in 3% agarose gel containing ethidium bromide and visualised under UV light.

CC homozygotes produce four DNA fragments of length 302 bp, 85bp, 42bp, and 8 bp. AA homozygotes produce five DNA fragments of 240 bp, 85 bp, 62 bp, 42bp, and 8 bp. Heterozygotes produce six DNA fragments of 302 bp, 240 bp, 85 bp, 62 bp, 42bp, and 8 bp.

IL-1β +3953 C/T Polymorphism

IL-1β gene exon 5 region (C allele):

gtctaatagt gtcaggtcca tgttcttagc caccccactc ccagcttcat ccctactggt
gttgtcatca gactttgacc gtatatgctc agtgtcctcc aagaaatcaa attttgccgc
ctcgcctcac gaggcctgcc cttctgattt tatacctaaa caacatgtgc tccacatttc

Taq I restriction site (dependant C +3953)

agaacctatc ttct**t**c**g**aca catgggataa cgaggcttat gtgcacgatg cacctgtacg

Taq I control site

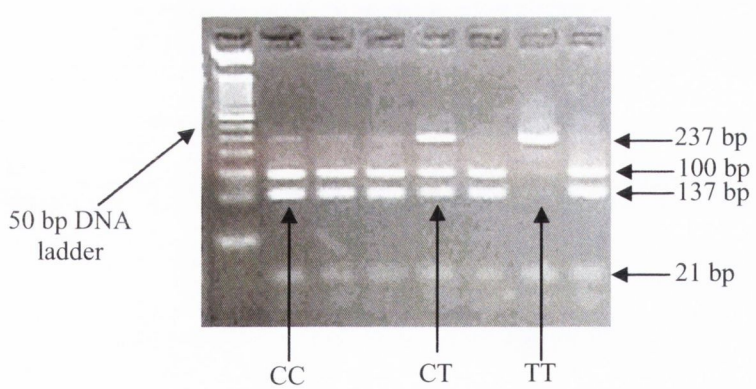
atcactgaac tgcacgctcc gggactcaca gcaaaaaagc ttggtgatgt ctggtcGata
tgaactgaaa gctctccacc tccagggaca ggatatggag caacaaggta aatggaaaca

Note: Base that changes from C to T at position +3953 is boxed. Uppercase G denotes base that is C in the gene sequence but G in the primer, creating a Taq I restriction site (control site) in the PCR product. Upstream and downstream primer sequences are highlighted in blue. Sequence NCBI Genbank accession number: M15840.

Taq I cuts at 5'- T▼CGA-3'

3'- AGC▲T-5'

Taq I digestion products of the IL-1β gene region containing the +3953 polymorphism:



Note: DNA bands were separated by electrophoresis in 3% agarose gel containing ethidium bromide and visualised under UV light.

CC homozygotes produce three DNA fragments of length 137 bp, 100 bp, and 21 bp. TT homozygotes produce two DNA fragments of 237 bp and 21 bp. Heterozygotes produce four DNA fragments of 237 bp, 137 bp, 100 bp, and 21 bp.

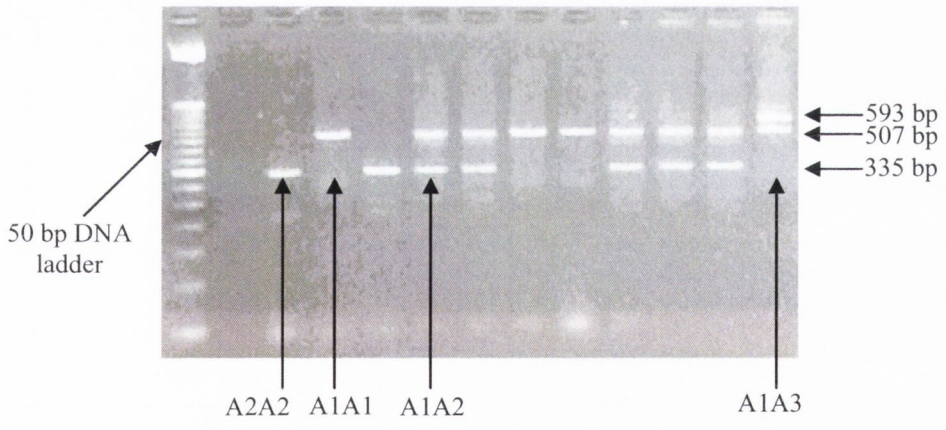
IL-1RN 86 bp VNTR

IL-1RN gene intron 2 region (A1 allele):

ggctcctggt ggagccccac tcatggcctt gttcattttc cctgcccctc agcaacactc
ctattgacct ggagcacagg t[atcctgggg aaagtgaggg aaatatggac atcacatgga
acaacatcca ggagactcag gcctctagga gtaactgggt agtgtgc|atc ctggggaaag
tgagggaaat atggacatca catggaacaa catccaggag actcaggcct ctaggagtaa
ctgggtagtg tgc|atcctgg ggaaagtgag ggaaatatgg acatcacatg gaacaacatc
caggagactc aggcctctag gagtaactgg gtagtgtgc|a tcctggggaa agtgagggaa
atatggacat cacatggaac aacatccagg agactcaggc ctctaggagt aactgggtag
tgtgc]ttggt ttaatcttct atttacctgc agaccaggaa gatgagacct ctctgccctt
ctgacctcgg gattttagtt ttgtggggac caggggagat agaaaaatac ccggggtctc
ttcattattg ctgcttcctc ttctattaac ctgaccctcc cctctgttct tccccagaaa
agatagatgt ggtacccatt gagcctcatg ctctgttctt gggaatccat ggagggaaga

Note: Tandem repeats separated by | . Upstream and downstream primer sequences are highlighted in blue. Sense primer from (Fang *et al*, 1999). Sequence NCBI Genbank accession number: X64532.

PCR products of the IL-1RN gene region containing variable numbers of the 86 bp tandem repeat:



Note: DNA bands were separated by electrophoresis in 2% agarose gel containing ethidium bromide and visualised under UV light.

The A1 allele, which contains 4 86 bp repeats, results in a DNA fragment of length 507 bp. The A2 allele, with 2 repeats, produces a 335 bp DNA fragament. The A3 allele, 5 repeats, gives a 593 bp DNA fragment. The rarer A4 (3 repeats) and A6 (6 repeats) alleles result in fragments of 421 bp and 679 bp respectively.

APPENDIX B (i)

Appendix B (i) Genes that are 2.5- to 2.99-fold upregulated in LPS treated THP1 cells compared to untreated cells

Gene Name	GenBank ID	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]	
		Untreated (Cy3)	LPS (Cy5)	Untreated (Cy3)	LPS (Cy5)	LPS/ Untreated	Untreated /LPS
Hypothetical protein	NM_015370	1004	2534	2.32	6.96	2.99	0.33
Wilms' tumour 1-associating protein (WTAP)	NM_004906	1252	3131	2.90	8.60	2.96	0.34
Tumor necrosis factor, alpha-induced protein 3 (TNF-αIP-3)	NM_006290	360	896	0.83	2.46	2.95	0.34
X transporter protein 3, isoform 1 (XT-3)	NM_020208	452	1111	1.05	3.05	2.92	0.34
Regulator of G-protein signalling 1 (RGS-1)	NM_002922	937	2305	2.17	6.33	2.92	0.34
Ferritin, heavy polypeptide 1 (FTH-1)	NM_002032	11621	28161	26.91	77.31	2.87	0.35
Prostaglandin-endoperoxide synthase 2 (PTGS-2)	NM_000963	304	731	0.70	2.01	2.85	0.35
Hypothetical protein	XM_071938	23766	56708	55.04	155.69	2.83	0.35
Chromosome 8 open reading frame 1 (C8orf-1)	NM_004337	529	1262	1.23	3.47	2.83	0.35
Ribosomal protein, large, P1 (RPLP-1)	NM_001003	25187	60077	58.33	164.93	2.83	0.35
Guanylate binding protein 2, interferon-inducible (GBP-2)	NM_004120	153 [b]	361	0.35	0.99	2.80	0.36
Ubiquitin A-52 residue ribosomal protein fusion product 1 (UBA-52)	NM_003333	20415	48130	47.28	132.14	2.79	0.36
Adipose differentiation-related protein (ADFP)	NM_001122	2636	6176	6.11	16.96	2.78	0.36
Ribosomal protein S25 (RPS25)	NM_001028	23786	55435	55.08	152.19	2.76	0.36
Syndecan binding protein (syntenin) (SDCBP)	NM_005625	937	2177	2.17	5.98	2.75	0.36
Prostaglandin E receptor 2 (subtype EP2), 53kDa (PTGER-2)	NM_000956	315	729	0.73	2.00	2.75	0.36
Ribosomal protein L10a (RPL10A)	NM_007104	22897	52891	53.02	145.21	2.74	0.37
Tropomyosin 4 (TPM-4)	NM_003290	2520	5815	5.84	15.96	2.74	0.37
Interferon gamma receptor 2 (interferon gamma transducer 1) (IFNGR-2)	NM_005534	913	2099	2.11	5.76	2.73	0.37

[a] Calculated by dividing the normalised signal intensity for LPS treated cells by that for untreated cells, and for untreated cells by LPS treated cells.
[b] Signal intensity below 'no expression' cut off point (Section 3.3.3), indicating no gene expression and thus unreliable fold difference in gene expression value.

Appendix B (i) continued Genes that are 2.5- to 2.99-fold upregulated in LPS treated THP1 cells compared to untreated cells

Gene Name	GenBank ID	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]	
		Untreated (Cy3)	LPS (Cy5)	Untreated (Cy3)	LPS (Cy5)	LPS/ Untreated	Untreated /LPS
Hypothetical protein	XM_052761	23874	54884	55.29	150.68	2.73	0.37
Similar to 40S ribosomal protein S3Aa (V-fos transformation effector protein)	XM_070421	23874	54624	55.29	149.97	2.71	0.37
Ribosomal protein L19 (RPL19)	NM_000981	19333	44205	44.77	121.36	2.71	0.37
Chymotrypsinogen B1 (CTRB-1)	NM_001906	886	1998	2.05	5.49	2.67	0.37
Ribosomal protein S20 (RPS20)	NM_001023	21167	47618	49.02	130.73	2.67	0.37
Annexin V (ANXA-5)	NM_001154	2497	5592	5.78	15.35	2.65	0.38
Clone FLB3816	NM_016415	65490	146104	151.66	401.12	2.64	0.38
Ribosomal protein l5 (RPl5)	NM_000969	25213	56145	58.39	154.14	2.64	0.38
Ribosomal protein S24, isoform C (RPS24)	NM_001026	8365	18532	19.37	50.88	2.63	0.38
Ribosomal protein L32 (RPL32)	NM_000994	20418	44923	47.28	123.33	2.61	0.38
Ribosomal protein S23 (RPS23)	NM_001025	22791	49465	52.78	135.80	2.57	0.39
Tissue inhibitor of metalloproteinase 1 precursor (TIMP-1)	NM_003254	1552	3350	3.59	9.20	2.56	0.39
ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit (ATP5E)	NM_006886	2109	4547	4.88	12.48	2.56	0.39
CDK4-binding protein p34SEI-1 (SEI-1)	NM_013376	592	1275	1.37	3.50	2.56	0.39
Ribosomal protein, large, P0 (RPLP0)	NM_053275	50814	109225	117.68	299.87	2.55	0.39
Zinc finger protein 267 (ZNF-267)	NM_003414	830	1782	1.92	4.89	2.54	0.39
Dual specificity phosphatase 2 (DUSP-2)	NM_004418	491	1049	1.14	2.88	2.53	0.39
Hypothetical protein	XM_071619	2078	4428	4.81	12.16	2.53	0.40
Ribosomal protein S16 (RPS16)	NM_001020	23328	49547	54.02	136.03	2.52	0.40
Destrin (actin depolymerizing factor) (DSTN)	NM_006870	492	1043	1.14	2.86	2.51	0.40

[a] Calculated by dividing the normalised signal intensity for LPS treated cells by that for untreated cells, and for untreated cells by LPS treated cells.

Appendix B (i) continued Genes that are 2.5- to 2.99-fold upregulated in LPS treated THP1 cells compared to untreated cells

Gene Name	GenBank ID	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]	
		Untreated (Cy3)	LPS (Cy5)	Untreated (Cy3)	LPS (Cy5)	LPS/ Untreated	Untreated /LPS
Ribosomal protein L13 (RPL13)	NM_033251	20715	43906	47.97	120.54	2.51	0.40
Similar to ferritin heavy chain (ferritin H subunit)	XM_060006	8375	17717	19.40	48.64	2.51	0.40
Solute carrier family 3, member 2 (SLC3A2)	NM_002394	1800	3798	4.17	10.43	2.50	0.40
Ribosomal protein S17 (RPS17)	NM_001021	17480	36894	40.48	101.29	2.50	0.40
Ribosomal protein L21 (gene or pseudogene) (RPL21)	NM_000982	16560	34914	38.35	95.85	2.50	0.40
Small nuclear ribonucleoprotein polypeptide G (SNRPG)	XM_015513	3092	6517	7.16	17.89	2.50	0.40
Ribosomal protein L23A (RPL23A)	NM_000984	17446	36732	40.40	100.84	2.50	0.40

[a] Calculated by dividing the normalised signal intensity for LPS treated cells by that for untreated cells, and for untreated cells by LPS treated cells.

APPENDIX B (ii)

Appendix B (ii) Genes that are expressed in untreated cells but show no expression in LPS-treated cells

Gene Name	GenBank ID	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]
		Untreated (Cy3)	LPS (Cy5)	Untreated (Cy3)	LPS (Cy5)	
Deleted in lymphocytic leukemia, 1 (DLEU-1)	NM_005887	803	261 [b]	1.86	0.72	1.94
Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein (ID-1)	NM_002165	764	343 [b]	1.77	0.94	1.84
Follicular lymphoma variant translocation 1 (FVT-1)	NM_002035	758	271 [b]	1.76	0.74	1.83
Solute carrier family 39 (zinc transporter), member 1 (SLC39A1)	NM_014437	756	191 [b]	1.75	0.52	1.82
Abhydrolase domain containing 2 (ABHD2)	NM_007011	725	201 [b]	1.68	0.55	1.75
Phosphatidylinositol-4-phosphate 5-kinase, type I, beta (PIP5K1B)	XM_015261	710	330 [b]	1.64	0.90	1.71
Dynactin 1, isoform 1 (DCTN-1)	NM_004082	710	337 [b]	1.64	0.92	1.71
Integrin alpha l precursor (ITGAL)	NM_002209	707	242 [b]	1.64	0.66	1.71
Putative protein P243 which interacts with transcription factor SP1	XM_056283	696	284 [b]	1.61	0.78	1.68
Hypothetical protein	NM_030578	691	310 [b]	1.60	0.85	1.67
Hypothetical protein	NM_022485	671	345 [b]	1.55	0.95	1.62
Placental protein 6 (PL6)	NM_007024	666	339 [b]	1.54	0.93	1.61
Protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), alpha isoform (PPP2R1A)	NM_014225	660	294 [b]	1.53	0.81	1.59
Pleckstrin homology domain containing, family B (evectins) member 2 (PLEKHB2)	NM_017958	654	266 [b]	1.51	0.73	1.58
Hypothetical protein	NM_024848	653	324 [b]	1.51	0.89	1.58
Vinexin beta (SH3-containing adaptor molecule-1) (SCAM-1)	NM_005775	647	297 [b]	1.50	0.82	1.56
IGF-II mRNA-binding protein 2 (IMP-2)	NM_006548	639	288 [b]	1.48	0.79	1.54

[a] Calculated by dividing the normalised signal intensity for untreated cells by 0.96. This was double the highest normalised intensity value displayed by an empty well and was taken as the cut-off point for no expression in the LPS-treated sample (0.96 corresponds to an intensity value of 350 bytes per pixel).

[b] Signal intensity below 'no expression' cut off point (Section 3.3.3), indicating no gene expression. This value can not therefore be used for calculation of gene expression ratio as results obtained would be unreliable due to slight fluctuations in background fluorescence between wells.

Appendix B (ii) continued Genes that are expressed in untreated cells but show no expression in LPS-treated cells

Gene Name	GenBank ID	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]
		Untreated (Cy3)	LPS (Cy5)	Untreated (Cy3)	LPS (Cy5)	
DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked (DDX3X)	NM_001356	625	287 [b]	1.45	0.79	1.51
Defensin, alpha 3, neutrophil-specific (DEFA3)	NM_005217	624	126 [b]	1.44	0.34	1.50
Ubiquitin-like, containing PHD and RING finger domains, 1 (UHRF1)	NM_013282	623	225 [b]	1.44	0.62	1.50
Adenosine monophosphate deaminase 2 (isoform l) (AMPD-2)	NM_004037	622	330 [b]	1.44	0.91	1.50
Chromosome 11 open reading frame 23 (C11orf23)	NM_018312	620	333 [b]	1.44	0.91	1.50

[a] Calculated by dividing the normalised signal intensity for untreated cells by 0.96. This was double the highest normalised intensity value displayed by an empty well and was taken as the cut-off point for no expression in the LPS-treated sample (0.96 corresponds to an intensity value of 350 bytes per pixel).

[b] Signal intensity below 'no expression' cut off point (Section 3.3.3), indicating no gene expression. This value can not therefore be used for calculation of gene expression ratio as results obtained would be unreliable due to slight fluctuations in background fluorescence between wells.

APPENDIX B (iii)

Appendix B (iii) Genes that are expressed in untreated cells but show no expression in PC-treated cells

Gene Name	GenBank ID	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]
		Untreated (Cy3)	LPS (Cy5)	Untreated (Cy3)	LPS (Cy5)	
Phosphatidylinositol-4-phosphate 5-kinase, type I, beta (PIP5K1B)	XM_015261	612	307 [b]	1.26	0.60	1.70
Deleted in lymphocytic leukemia, 1 (DLEU-1)	NM_005887	599	191 [b]	1.23	0.37	1.66
Defensin, alpha 3, neutrophil-specific (DEFA3)	NM_005217	587	149 [b]	1.21	0.29	1.63
PRO-1085 protein, isoform 1	AL354776	569	165 [b]	1.17	0.32	1.58
Mannan-binding lectin serine protease 2 (MASP-2)	NM_006610	549	283 [b]	1.13	0.55	1.52

[a] Calculated by dividing the normalised signal intensity for untreated cells by 0.74. This was double the highest normalised intensity value displayed by an empty well and was taken as the cut-off point for no expression in the PC-treated sample (0.74 corresponds to an intensity value of 380 bytes per pixel).

[b] Signal intensity below 'no expression' cut off point (Section 3.3.4), indicating no gene expression. This value can not therefore be used for calculation of gene expression ratio as results obtained would be unreliable due to slight fluctuations in background fluorescence between wells.

APPENDIX B (iv)

Appendix B (iv) Genes that are 2.5- to 2.99-fold upregulated in LPS treated THP1 cells compared to LPS + PC treated cells

Gene Name	GenBank ID	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]	
		L + PC (Cy3)	LPS (Cy5)	L + PC (Cy3)	LPS (Cy5)	LPS/ L + PC	L + PC/ LPS
Growth arrest and dna-damage-inducible, alpha (GADD-45α)	NM_001924	1303	4215	2.74	8.12	2.97	0.34
Wilms' tumour 1-associating protein (WTAP)	NM_004906	2650	8277	5.56	15.94	2.87	0.35
Annexin A1 (ANX-A1)	NM_000700	3093	9592	6.49	18.48	2.85	0.35
Similar to ferritin heavy chain (ferritin H subunit)	XM_060006	14551	45016	30.55	86.70	2.84	0.35
ATPase, Ca^{++} transporting, plasma membrane 1 (ATP2B1)	NM_001682	1216	3749	2.55	7.22	2.83	0.35
Hypothetical protein	XM_018436	599	1840	1.26	3.54	2.82	0.35
Nuclear factor of kappa light polypeptide gene enhancer in B cells 1 (p105) (NFκB-1)	NM_003998	518	1589	1.09	3.06	2.82	0.35
Vimentin (VIM)	NM_003380	3254	9947	6.83	19.16	2.80	0.36
Beta actin	X63432	40127	122428	84.23	235.81	2.80	0.36
Lymphocyte cytosolic protein 1 (L-plastin) (LCP-1)	NM_002298	2754	8298	5.78	15.98	2.76	0.36
BCL2-related protein A1 (BCL2A1)	NM_004049	614	1848	1.29	3.56	2.76	0.36
Regulator of G-protein signalling 1 (RGS-1)	NM_002922	1408	4224	2.95	8.14	2.75	0.36
Prostaglandin E receptor 2 (subtype EP2), 53kDa (PTGER2)	NM_000956	546	1629	1.15	3.14	2.74	0.37
Tumor necrosis factor, alpha-induced protein 6 (TNFAIP-6)	NM_007115	563	1664	1.18	3.20	2.71	0.37
Regulator of G-protein signalling 2, 24kDa (RGS-1)	NM_002923	982	2883	2.06	5.55	2.69	0.37
Hypothetical protein	XM_071456	2505	7342	5.26	14.14	2.69	0.37
Beta actin	X63432	64881	187018	136.20	360.21	2.64	0.38
Chymotrypsinogen B1 (CTRB-1)	NM_001906	1101	3155	2.31	6.08	2.63	0.38

[a] Calculated by dividing the normalised signal intensity for LPS treated cells by that for LPS + PC treated cells, and for LPS + PC treated cells by LPS treated cells.

Appendix B (iv) continued Genes that are 2.5- to 2.99-fold upregulated in LPS treated THP1 cells compared to LPS + PC treated cells

Gene Name	GenBank ID	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]	
		L + PC (Cy3)	LPS (Cy5)	L + PC (Cy3)	LPS (Cy5)	LPS/ L + PC	L + PC/ LPS
Hypothetical protein	XM_016170	65472	185056	137.44	356.43	2.59	0.39
Lipase, hormone-sensitive (LIPE)	NM_005357	1402	3937	2.94	7.58	2.58	0.39
Hypothetical protein	NM_018277	434	1212	0.91	2.33	2.56	0.39
Nuclease sensitive element binding protein 1 (NSEP-1)	NM_004559	65465	182651	137.42	351.80	2.56	0.39
Dual specificity phosphatase 2 (DUSP-2)	NM_004418	606	1680	1.27	3.24	2.54	0.39
Insulin-like growth factor binding protein 3 (IGFBP-3)	NM_000598	567	1558	1.19	3.00	2.52	0.40
V-ets erythroblastosis virus E26 oncogene homolog 2 (avian) (ETS-2)	NM_005239	494	1344	1.04	2.59	2.50	0.40

[a] Calculated by dividing the normalised signal intensity for LPS treated cells by that for LPS + PC treated cells, and for LPS + PC treated cells by LPS treated cells.
[b] Signal intensity below 'no expression' cut off point (Section 3.3.5), indicating no gene expression and thus unreliable fold difference in gene expression value.

APPENDIX B (v)

Appendix B (v) Genes that are expressed in LPS + PC-treated cells but show no expression in LPS-treated cells

Gene Name	GenBank ID	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]
		LPS + PC (Cy3)	LPS (Cy5)	LPS + PC (Cy3)	LPS (Cy5)	
Hypothetical protein	XM_071552	564	259 [b]	1.18	0.50	1.97
Zinc finger protein 238 (ZNF-238)	NM_006352	558	281 [b]	1.17	0.54	1.95
Rap2 interacting protein x (RIPX)	NM_014961	546	179 [b]	1.15	0.34	1.91
Protein tyrosine phosphatase, non-receptor type 22 (lymphoid) (PTPN22)	NM_015967	535	259 [b]	1.12	0.50	1.87
Zinc finger protein 237 protein (ZNF-237)	AJ133352	529	282 [b]	1.11	0.54	1.85
Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 3 (SPOCK3)	NM_016950	529	106 [b]	1.11	0.20	1.85
Interleukin-1 receptor-associated kinase 4 (IRAK4)	NM_016123	528	263 [b]	1.11	0.51	1.85
Crystallin, gamma B (CRYGB)	NM_005210	527	108 [b]	1.11	0.21	1.84
Similar to adenosine A1 receptor (H. sapiens)	XM_001687	521	155 [b]	1.09	0.30	1.82
Dihydropyrimidinase-like 3 (DPYSL3)	NM_001387	505	189 [b]	1.06	0.36	1.77
Sine oculis homeobox (Drosophila) homologue 1 (SIX-1)	NM_005982	494	209 [b]	1.04	0.40	1.73
Homeo box A5 (HOXA5)	NM_019102	488	137 [b]	1.03	0.26	1.71
Hypothetical protein	XM_045675	483	217 [b]	1.01	0.42	1.69
Potassium voltage-gated channel, delayed-rectifier, subfamily S, member 3 (KCNS-3)	NM_002252	481	240 [b]	1.01	0.46	1.68
Serine protease inhibitor, kazal type, 5 (SPINK-5)	NM_006846	477	101 [b]	1.00	0.19	1.67
KIAA0445 gene product	NM_014675	475	224 [b]	1.00	0.43	1.66
Keratin 12 (KRT-12)	NM_000223	473	245 [b]	0.99	0.47	1.66

[a] Calculated by dividing the normalised signal intensity for LPS + PC-treated cells by 0.6. This was double the highest normalised intensity value displayed by an empty well and was taken as the cut-off point for no expression in the LPS-treated sample (0.6 corresponds to an intensity value of 312 bytes per pixel).

[b] Signal intensity below 'no expression' cut off point (Section 3.3.5), indicating no gene expression. This value can not therefore be used for calculation of gene expression ratio as results obtained would be unreliable due to slight fluctuations in background fluorescence between wells.

Appendix B (v) continued Genes that are expressed in LPS + PC-treated cells but show no expression in LPS-treated cells

Gene Name	GenBank ID	Signal Intensity (bytes per pixel)		Normalised Signal Intensity		Fold Difference [a]
		LPS + PC (Cy3)	LPS (Cy5)	LPS + PC (Cy3)	LPS (Cy5)	
Calcyphosine (CAPS)	NM_004058	473	184 [b]	0.99	0.35	1.65
Uncharacterized bone marrow protein BM039 (BM039)	NM_018455	464	192 [b]	0.97	0.37	1.62
A disintegrin and metalloproteinase domain 30, isoform 2 preproprotein (ADAM-30)	NM_020334	461	87 [b]	0.97	0.17	1.61
Chromosome 14 open reading frame 103 (C14orf103)	NM_018036	456	226 [b]	0.96	0.44	1.59
Rho-associated, coiled-coil containing protein kinase 2 (ROCK-2)	NM_004850	454	170 [b]	0.95	0.33	1.59
zinc finger protein 287 (ZNF-287)	NM_020653	454	158 [b]	0.95	0.30	1.59
Phosphodiesterase 5A, isoform 3 (PDE5A)	NM_033437	452	209 [b]	0.95	0.40	1.58
Pleckstrin 2 (mouse) homologue (PLEK-2)	NM_016445	451	230 [b]	0.95	0.44	1.58
Membrane-associated protein 17 (MAP17)	NM_005764	445	175 [b]	0.93	0.34	1.56
Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 7 (SERPINB7)	NM_003784	443	136 [b]	0.93	0.26	1.55
Hypothetical protein	NM_018532	440	141 [b]	0.92	0.27	1.54
Angiopoietin 1 (ANGPT-1)	NM_001146	436	114 [b]	0.91	0.22	1.52
G protein-coupled receptor 52 (GPR-52)	NM_005684	436	113 [b]	0.91	0.22	1.52
MHC class I polypeptide-related sequence B (MICB)	NM_005931	435	199 [b]	0.91	0.38	1.52
ELK4, ETS-domain protein (SRF accessory protein 1) (ELK4)	NM_001973	435	163 [b]	0.91	0.31	1.52
Hypothetical protein	NM_024639	434	196 [b]	0.91	0.38	1.52
Formyl peptide receptor 1 (FPR-1)	NM_002029	431	121 [b]	0.90	0.23	1.51
Activin A type I receptor precursor (ACVR-1)	NM_001105	431	159 [b]	0.90	0.31	1.51

[a] Calculated by dividing the normalised signal intensity for LPS + PC-treated cells by 0.6. This was double the highest normalised intensity value displayed by an empty well and was taken as the cut-off point for no expression in the LPS-treated sample (0.6 corresponds to an intensity value of 312 bytes per pixel).

[b] Signal intensity below 'no expression' cut off point (Section 3.3.5), indicating no gene expression. This value can not therefore be used for calculation of gene expression ratio as results obtained would be unreliable due to slight fluctuations in background fluorescence between wells.

APPENDIX C

TLR4 Gene DNA: complete coding sequence (AF177765)

12601 tagacctgtc cctgaaccct .....exon 4..... cagtcgtgct ggtatcatct

14281 tcattgtcct gcagaaggtg gagaagaccc tgctcaggca gcaggtggag ctgtaccgcc

14341 ttctcagcag gaacacttac ctggagtggg aggacagtgt cctggggcgg cacatcttct

14401 ggagacgact cagaaaagcc ctgctggatg gtaaatcatg gaatccagaa ggaacagtgg

Stop Codon 3' UTR ⟶

14461 gtacaggatg caattggcag gaagcaacat ctatctga|ag aggaaaaata aaaacctcct

14521 gaggcatttc .....3' UTR..... agacacagat ggctgggatc cctcccctgt

⟵ exon 4

15601 acccttctca ctgccaggag aacta|cgtgt gaaggtattc aaggcaggga gtatacattg

15661 ctgtttcctg ttgggcaatg ctccttgacc acattttggg aagagtggat gttatcattg

15721 agaaaacaat⟶

TLR4 Gene mRNA Sequence (XM_057452)

⟵ 5' UTR Start Codon

1 ⟵ gccac tgctgctcac agaagcagtg aggatgatgc cagg|atgatg tctgcctcgc

61 gcctggctgg gactctgatc ccagccatgg ccttcctctc ctgcgtgaga ccagaaagct

⟵ exon 1 exon 2 (alternative – results in truncated TLR4 protein) ⟶

121 gggagccctg cgtggag|act tggccctaaa ccacacagaa gagctggcat gaaacccaga

181 gctttcagac tccggagcct cagcccttca ccccgattcc attgcttctt gctaaatgct

⟵ exon 2 exon 3 ⟶

241 gccgttttat cacggag|gtg gttcctaata ttacttatca atgcatggag ctgaatttct

301 acaaaatccc cgacaacctc cccttctcaa ccaagaacct ggacctgagc tttaatcccc

361 tgaggcattt aggcagctat agcttcttca gtttcccaga actgcaggtg ctggatttat

⟵ exon 3 exon 4 ⟶

421 ccag|gtgtga aatccagaca attgaagatg gggcatatca gagcctaagc cacctctcta

481 ccttaatatt gacaggaaac cccatccaga gtttagccct gggagccttt tctggactat

541 caagtttaca gaagctggtg gctgtggaga caaatctagc atctctagag aacttcccca

601 ttggacatct caaaactttg aaagaactta atgtggctca caatcttatc caatctttca

661 aattacctga gtatttttct aatctgacca atctagagca cttggacctt tccagcaaca

721 agattcaaag tatttattgc acagacttgc gggttctaca tcaaatgccc ctactcaatc

781 tctctttaga cctgtccctg aaccctatga actttatcca accaggtgca tttaaagaaa

841 ttaggcttca taagctgact ttaagaaata attttgatag tttaaatgta atgaaaactt

901 gtattcaagg tctggctggt ttagaagtcc atcgtttggt tctgggagaa tttagaaatg

961 aaggaaactt ggaaaagttt gacaaatctg ctctagaggg cctgtgcaat ttgaccattg

1021 aagaattccg attagcatac ttagactact acctcgatga tattattgac ttatttaatt

1081 gtttgacaaa tgtttcttca ttttccctgg tgagtgtgac tattgaaagg gtaaaagact

1141 tttcttataa tttcggatgg caacatttag aattagttaa ctgtaaattt ggacagtttc

1201 ccacattgaa actcaaatct ctcaaaaggc ttactttcac ttccaacaaa ggtgggaatg

1261 ctttttcaga agttgatcta ccaagccttg agtttctaga tctcagtaga aatggcttga

1321 gtttcaaagg ttgctgttct caaagtgatt ttgggacaac cagcctaaag tatttagatc

1381 tgagcttcaa tggtgttatt accatgagtt caaacttctt gggcttagaa caactagaac

1441 atctggattt ccagcattcc aatttgaaac aaatgagtga gttttcagta ttcctatcac

1501 tcagaaacct catttacctt gacatttctc atactcacac cagagttgct ttcaatggca

1561 tcttcaatgg cttgtccagt ctcgaagtct tgaaaatggc tggcaattct ttccaggaaa

1621 acttccttcc agatatcttc acagagctga gaaacttgac cttcctggac ctctctcagt

1681 gtcaactgga gcagttgtct ccaacagcat ttaactcact ctccagtctt caggtactaa

1741 atatgagcca caacaacttc ttttcattgg atacgtttcc ttataagtgt ctgaactccc

1801 tccaggttct tgattacagt ctcaatcaca taatgacttc caaaaaacag gaactacagc

1861 attttccaag tagtctagct ttcttaaatc ttactcagaa tgactttgct tgtacttgtg

1921 aacaccagag tttcctgcaa tggatcaagg accagaggca gctcttggtg gaagttgaac

1981 gaatggaatg tgcaacacct tcagataagc agggcatgcc tgtgctgagt ttgaatatca

2041 cctgtcagat gaataagacc atcattggtg tgtcggtcct cagtgtgctt gtagtatctg

2101 ttgtagcagt tctggtctat aagttctatt ttcacctgat gcttcttgct ggctgcataa

2161 agtatggtag aggtgaaaac atctatgatg cctttgttat ctactcaagc caggatgagg

2221 actgggtaag gaatgagcta gtaaagaatt tagaagaagg ggtgcctcca tttcagctct

2281 gccttcacta cagagacttt attcccggtg tggccattgc tgccaacatc atccatgaag

2341 gtttccataa aagccgaaag gtgattgttg tggtgtccca gcacttcatc cagagccgct

2401 ggtgtatctt tgaatatgag attgctcaga cctggcagtt tctgagcagt cgtgctggta

2461 tcatcttcat tgtcctgcag aaggtggaga agaccctgct caggcagcag gtggagctgt

2521 accgccttct cagcaggaac acttacctgg agtgggagga cagtgtcctg gggcggcaca

2581 tcttctggag acgactcaga aaagccctgc tggatggtaa atcatggaat ccagaaggaa

Stop Codon 3' UTR →

2641 cagtgggtac aggatgcaat tggcaggaag caacatctat ctga|agagga aaaataaaaa

2701 cctcctgagg catttcttgc ⟶