NOVEL ROLES FOR ELAFIN IN THE INNATE IMMUNE RESPONSE

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DECLARATION

I hereby declare that this thesis has been composed entirely by myself and that no part of this work has been submitted for any other degree or professional qualification. All work presented in this thesis was executed by myself except where otherwise acknowledged.

Jonathan W. McMichael

ABSTRACT

The innate immune response to bacterial products plays a critical role in host defence against infection, yet this response can be of benefit or detriment to the host depending on its magnitude. Lipopolysaccharide (LPS) or endotoxin, the major component of the outer membrane of Gram-negative bacteria, is the archetypal bacterial signal that activates innate immunity. LPS provides a potent and pleiotropic stimulus for immune cells and has been implicated in the pathogenesis of various clinical conditions. Most significantly, exposure to excessive levels of LPS in the circulation can lead to Gram-negative septic shock, a clinical syndrome characterised by fever, disseminated intravascular coagulation, multiple organ failure, and potentially death. Thus, strategies aimed at modulating the effects of LPS on the innate immune response are desirable.

Elafin is a cationic, low molecular weight elastase inhibitor produced locally in the respiratory tract, in the skin and at mucosal sites in many tissues. Several biochemical characteristics of elafin, such as low molecular weight, cationicity, heavy disulphide bonding and tissue distribution at mucosal sites, suggested that it may possess anti-endotoxin properties similar to defensins and other cationic antimicrobial peptides.

Here we have demonstrated that elafin binds directly to LPS of both smooth-form and rough-form type. This binding was shown to occur within the conserved lipid A portion of the LPS molecule, and binding of elafin to LPS inhibited subsequent interaction of LPS with LPS-binding protein (LBP). Moreover, both terminal domains of elafin were shown to bind LPS and preclude this interaction.

Furthermore, elafin inhibited TNF-α secretion from LPS-stimulated macrophages in serum-containing conditions *in vitro*, but enhanced TNF-α secretion in serum-free milieu. These findings suggest that extracellular elafin may play divergent roles in the innate immune response to LPS depending on the site of infection, for example serum-rich bloodstream or serum-deficient mucosal sites. A

replication-deficient adenovirus vector encoding human elafin cDNA (Ad-elafin) was used to extend these studies to demonstrate that elafin may also act intracellularly to dampen macrophage responses to LPS.

This LPS-modulatory activity prompted us to study the antimicrobial properties of elafin. Ad-elafin infection of primary murine tracheal epithelial cells *ex vivo* conferred antimicrobial activity against *Staphylococcus aureus*, but compromised the inherent ability of cells to kill *Pseudomonas aeruginosa*.

In summary, elafin may play a role in innate immunity by modulating host responses to Gram-negative bacterial LPS. Elafin could function to enhance immune responses in sites of local inflammation, such as the airways, but to down-regulate potentially deleterious responses to LPS in the circulation.

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ABBREVIATIONS

aa	Amino acid
Ad	Adenovirus
Ad-elafin	Adenovirus encoding human elafin cDNA
Ad-lacZ	Adenovirus encoding the lacZ gene
AP-1	Activating protein-1
APS	Ammonium persulphate
ASL	Airway surface liquid
BAL	Bronchoalveolar lavage
BPI	Bactericidal/permeability-increasing protein
BSA	Bovine serum albumin
C1705	*Staphylococcus aureus* strain C1705
$CaCl_2$	Calcium chloride
CaPi	Calcium phosphate
CAR	Coxsackie-adenovirus receptor
CARD	Caspase-recruitment domain
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
cfu	Colony forming units
CHO	Chinese hamster ovary
CMV	Cytomegalovirus
COOH-	Carboxy-
DAB	α-γ-diaminobutyric acid
DC	Dendritic cell
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
ECL	Enhanced chemiluminescence

EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
GPI	Glycosylphosphatidylinositol
H. influenzae	*Haemophilus influenzae*
HBD	Human β-defensin
HD	Human defensin
HEK	Human embryonic kidney
HNE	Human neutrophil elastase
HNP	Human neutrophil peptide
HRP	Horseradish peroxidase
HSP	Heat-shock protein
IκB	Inhibitor of κB
IFN	Interferon
IKK	Inhibitor of κB kinase
IL	Interleukin
IRAK	Interleukin-1 receptor-associated kinase
IRF3	Interferon-regulatory factor 3
J1385	*Pseudomonas aeruginosa* strain J1385
JNK	c-Jun N-terminal kinase
K. pneumoniae	*Klebsiella pneumoniae*
kDa	Kilodaltons
Kdo	2-keto-3-deoxyoctulosonic acid
LAL	Limulus amoebocyte lysate
LBP	Lipopolysaccharide-binding protein
LDS	Lithium dodecyl sulphate
LOS	Lipo-oligosaccharide
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LTA	Lipoteichoic acid
mAb	Monoclonal antibody
Mal	MyD88-adapter-like

MAPK	Mitogen-activated protein kinase
mCD14	Membrane-bound CD14
MCMV	Murine cytomegalovirus
MCP	Monocyte chemoattractant protein
MD-2	Myeloid differentiation protein-2
MEEM	Minimum Essential Eagle's Medium
MIF	Macrophage migration inhibitory factor
MIP	Macrophage inflammatory protein
moi	Multiplicity of infection
MPC	Multicatalytic proteinase complex
MRSA	Methicillin-resistant *Staphylococcus aureus*
MyD88	Myeloid differentiation factor 88
N. meningitidis	*Neisseria meningitidis*
NF	Nuclear factor
NH_2-	Amino-
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
O-	O-polysaccharide antigen
O.D.	Optical density
P. aeruginosa	*Pseudomonas aeruginosa*
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PAO1	*Pseudomonas aeruginosa* strain PAO1
PBS	Phosphate buffered saline
p*I*	Isoelectric pH
PIA	*Pseudomonas* Isolation Agar
PRR	Pattern-recognition receptor
RNA	Ribonucleic acid
RSV	Respiratory syncytial virus
S. aureus	*Staphylococcus aureus*
S.E.	Standard error
sCD14	Soluble CD14

SD	Standard deviation
SDS	Sodium dodecyl sulphate
SLPI	Secretory leukocyte proteinase inhibitor
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF	Transforming growth factor
TICAM-1	TIR-containing adapter molecule-1
TIR	Toll/interleukin-1 receptor
TIRAP	TIR domain-containing adapter protein
TLR	Toll-like receptor
TMB	3,3',5'5-tetramethylbenzidine
TNF	Tumour necrosis factor
TRAF6	TNF receptor-associated factor 6
TRIF	TIR-domain containing adapter inducing IFN-β
USG	Ultroser G medium
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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INTRODUCTION

1.1. OVERVIEW

The immune system detects and eliminates invading pathogenic microorganisms by discriminating between self and infectious non-self. In mammals, the immune system can be segregated into two branches: the innate immune response and the adaptive immune response. Adaptive immunity relies on gene rearrangement and clonal expansion upon detection of specific antigens of the invading pathogen; this arm of the immune response is mediated by the activity of T and B cells, which engage non-self via antigen receptors to generate antigen-specific effector cells that specifically target the pathogen, and memory cells that prevent subsequent infection with the same organism. While the effectors of adaptive immunity may take weeks to generate, innate immune responses are mobilised within hours of exposure to a pathogen. Innate immune responses are therefore largely responsible for containing and limiting the spread of infection, and stimulating the adaptive immune response. Innate immunity is not pathogen-specific as it does not involve clonal expansion of antigen-specific lymphocytes, but rather relies on recognition of conserved molecular patterns exhibited by invading microorganisms.

Of particular importance in this regard is lipopolysaccharide (LPS) or endotoxin, a ubiquitous component of the outer membrane of Gram-negative bacteria. LPS is a potent agonist of the innate immune system. The rapid response against LPS can be of benefit to the host in local sites of infection and in moderate levels by promoting inflammation and priming the immune system to eradicate the invading pathogens; however, an excessive or systemic response to LPS (for example when LPS enters the bloodstream) can lead to sepsis, a systemic inflammatory condition characterised by multiple organ failure, shock and potentially death. The discovery of novel strategies aimed at modulating the inflammatory response to LPS are therefore of great interest.

One potential approach to addressing therapeutically the pathophysiological sequelae induced by LPS is to target this molecule directly, by using an agent capable of binding to it and sequestering its agonistic activities. In this regard, cationic antimicrobial peptides of the innate immune system are of particular interest, since they can interact with LPS and suppress many of its biological effects.

Our group is interested in the activities of elafin, a low molecular weight proteinase inhibitor (or 'four-disulphide core' protein) produced locally in the respiratory tract, in the skin and at mucosal sites in many tissues, that can exert antimicrobial activity against Gram-positive and Gram-negative bacterial pathogens. Several biochemical characteristics of elafin, such as low molecular weight, cationicity, heavy disulphide bonding and tissue distribution at mucosal sites, suggested that it may possess LPS-binding properties similar to those described for other antimicrobial peptides. Moreover, the low molecular weight anti-proteinase SLPI (secretory leukocyte proteinase inhibitor), with which elafin shares sequence homology, has been shown to bind to LPS and modulate its activity.

The work described in this thesis was thus stimulated by the following questions: Firstly, can the low molecular weight proteinase inhibitor elafin bind directly to Gram-negative LPS? If so, how does this interaction affect the ability of LPS to activate immune cells? Finally, can genetic augmentation of elafin modulate cellular responses to LPS and whole bacteria?

Against this background, the following sections will provide a brief overview of innate immunity, before going on to discuss the structure and activities of LPS, with emphasis on the pathophysiology of sepsis. These sections will be followed by a discussion of major components of the innate immune response, with particular reference to those involved in responses to LPS (such as the mechanisms of LPS recognition, Toll-like receptors (TLRs) and cationic antimicrobial peptides). Finally, endogenous proteinase inhibitors will be described in the latter part of this chapter, with special emphasis on the functions and properties of the four-disulphide core protein, elafin.

1.2. INNATE IMMUNITY

The innate immune system is a phylogenetically conserved, ancient mechanism of host defence that is present in virtually every multicellular organism, from plants to humans (reviewed by Medzhitov and Janeway, 2000). In invertebrates, innate immunity is the only mechanism of defence. By contrast with adaptive immunity, innate immune recognition is mediated by germline-encoded receptors, meaning that the specificity of each receptor is genetically pre-determined. These receptors have evolved by natural selection to possess defined specificities for infectious microorganisms. Such receptors are known as pattern-recognition receptors (PRRs), which identify highly conserved molecules present in bacteria and other pathogens. PRRs may be strategically expressed on cells that are first to encounter pathogens during infection, such as surface epithelia, and on effector cells of the innate immune system, such as macrophages and dendritic cells, which may act as antigen-presenting cells (APCs) for activation of acquired immune responses (Medzhitov and Janeway, 1997). The structures recognised by PRRs are known as pathogen-associated molecular patterns (PAMPs); prominent examples of PAMPs include bacterial LPS, lipoteichoic acid, peptidoglycan, flagellin, bacterial (CpG) DNA, lipoarabinomannans, phosphoglycolipids and viral double-stranded RNA (Medzhitov and Janeway, 2000; Singh *et al.*, 2003).

Although these molecular targets are structurally distinct, all PAMPs share a number of common features. Firstly, they are usually only expressed by microbial pathogens and are therefore distinct from self-antigens. Secondly, PAMPs demonstrate little variation among microorganisms of a given class. For example, all Gram-negative bacteria possess a form of LPS in their outer membrane. Finally, these patterns are generally essential components for the survival or pathogenicity of the microbe, and are thus not prone to antigenic variability.

Functionally, pattern-recognition receptors can be divided into three classes: secreted receptors (such as collectins), which function as opsonins by binding to microbial cell walls and marking them for recognition by the complement system and for phagocytosis (reviewed by Epstein *et al.*, 1996; Hickling *et al.*, 2004);

endocytic receptors (such as the macrophage mannose receptor and the macrophage scavenger receptor), which mediate uptake and delivery of the pathogen into lyosomes for destruction, and subsequent antigen presentation via major-histocompatibility-complex (MHC) molecules on the cell surface (Fraser *et al.*, 1998; Thomas *et al.*, 2000); and signalling receptors (such as the Toll-like receptor (TLR) family and NOD (nucleotide-binding oligomerization domain) proteins), which recognise PAMPs and activate intracellular signal-transduction pathways, leading to expression of a variety of immune-response genes (reviewed by Takeda *et al.*, 2003 and Inohara and Nunez, 2003, respectively). Table 1 describes the protein families believed to play a central role in forming PRRs.

A wealth of recent work has highlighted the prominent role played by the TLR family of receptors in the recognition of infectious microorganisms, and the subsequent initiation of immune and inflammatory responses. Moreover, TLR signalling pathways have been shown to be crucial in regulating cellular responses to LPS. Prior to examining the roles played by TLRs in the innate immune response and particularly in LPS recognition, it seems prudent to begin by discussing the structure of LPS and its function in activation of innate immunity.

Protein family (PRR)	Site of expression	Example	Ligands (PAMPs)	Known functions
C-type lectins				
Humoral	Plasma protein, pulmonary surfactant	Collectins (surfactant protein A/ D, MBL)	Bacterial and viral carbohydrates	Opsonisation, activation of complement (lectin pathway)
Cellular	Macrophages, dendritic cells	Macrophage C-type lectin	GalNAc receptor	Induces macrophage tumouricidal activity
	Macrophages, dendritic cells	Macrophage mannose receptor	Terminal mannose	Phagocytosis
	Macrophages, dendritic cells	DEC 205	Terminal mannose	Phagocytosis
	NK cells	NKR-P1	Carbohydrates	Cytolytic function, IFN-γ secretion
Leucine-rich proteins	Macrophages, epithelial cells	CD14	LPS	Signals cells
	Various cell types	TLRs	Various	Cell signalling & activation
	Various cell types	NOD proteins	LPS, peptidoglycan	Cell signalling & activation
Scavenger receptors	Macrophages	Macrophage scavenger receptor	Bacterial cell walls	Phagocytosis
Pentraxins	Plasma protein	C-reactive protein	Phosphatidyl choline	Opsonise, activate complement
	Plasma protein	Serum amyloid P	Bacterial cell walls	Opsonise, activate complement
Lipid transferases	Plasma protein	LBP	LPS, other LS	Bind LPS, transfer to CD14
	Plasma protein	BPI	LPS, other LS	Bactericidal activity, LPS neutralisation
Integrins	Macrophages, dendritic, NK, T cells	CD11b,c/CD18	LPS	Signal cells, phagocytosis

Table 1. Pattern recognition molecules (PRRs) of the innate immune system, and their associated PAMPs.
MBL, mannan-binding lectin; GalNAc, N-acetylgalactosamine; LPS, lipopolysaccharide; TLRs, Toll-like receptors; NOD, nucleotide-binding oligomerization domain; LBP, LPS-binding protein; BPI, bactericidal/permeability-increasing protein; LS, liposaccharide; PAMPs, pathogen-associated molecular patterns. Adapted from Medzhitov and Janeway (1997).

1.3. BACTERIAL LIPOPOLYSACCHARIDES

1.3.1. Overview of LPS

Bacterial lipopolysaccharides (LPS) or endotoxins are the major outer surface membrane components of Gram-negative bacteria, and are extremely powerful stimulators of the innate immune system. LPS is vital to both the structural and functional integrity of the Gram-negative outer membrane, and is expressed ubiquitously (in some form) by all Gram-negative bacteria (Erridge *et al*, 2002). Figure 1 provides a schematic representation of the location of LPS in the Gram-negative bacterial cell wall.

As a primary target of innate immunity, recognition of LPS by cells such as monocytes and macrophages provides the host with a rapid and vigorous response to Gram-negative infection. Endotoxins can affect the functions of numerous cells and organs, alter blood enzyme levels, modify sugar, fat and protein metabolism, raise or lower body temperature, induce blood coagulation, tissue damage and organ failure, and in extreme cases can lead to death of the host (Haeffner-Cavaillon *et al.*, 1998). The majority of these effects result from the ability of LPS to induce the release of a gamut of pro-inflammatory mediators, such as tumour necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β. As mentioned in section 1.1., the reaction of the immune system to the presence of LPS can be of benefit or detriment to the host, depending on the site or magnitude of the response. Agonistic LPS may either induce an increase in the general immune resistance against microbial infections, or evoke serious pathologic symptoms such as those observed during sepsis syndromes related to Gram-negative bacteraemia and endotoxaemia (Alexander and Rietschel, 2001).

With this is mind, the following sections will discuss the general structural features of the LPS molecule, the relationship of these features to biological activity of the holo-molecule, and the consequences of an over-vigorous or unregulated immune response to LPS *in vivo*.

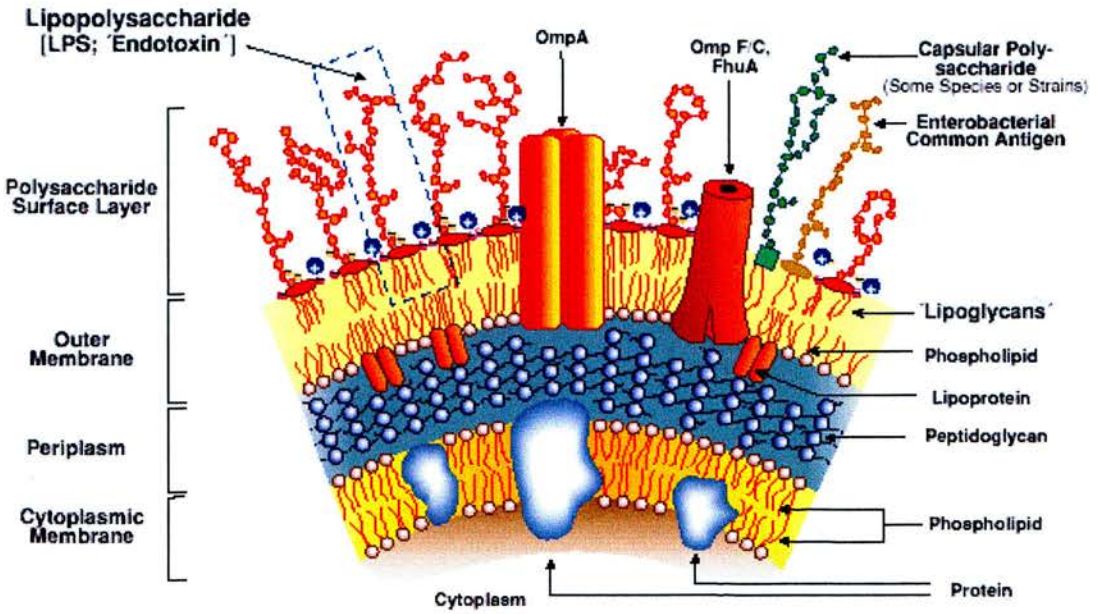


Figure 1. Cell wall architecture of Gram-negative bacteria.

Gram-negative bacterial cell walls are characterised by the presence of two lipid bilayers, the outer and inner (or cytoplasmic) membranes, which are separated by the periplasmic space containing peptidoglycan. In contrast, Gram-positive bacteria do not possess an outer membrane in their cell wall, and do not elaborate LPS. In Gram-negative bacteria, the lipid portion of the outward directed membrane leaflet is formed predominantly by lipid A of LPS molecules, which make up around 75% of total membrane surface and form contacts to integral outer membrane proteins (such as porins Omp A or Omp F/C, or the bacterial ferric hydroximate uptake receptor FhuA). In addition to LPS, the outer membrane polysaccharide coat of enterobacteria is formed by the enterobacterial common antigen and, in some species and strains, by capsular polysaccharides. In the physiological situation, divalent cations are tightly associated to the highly anionic membrane-proximal LPS moieties. The inner layer of the outer membrane, as well as the lipid layers of the cytoplasmic membrane, are predominantly composed of phospholipids. The membrane-inserted acyl moieties of bacterial lipoproteins also anchor the outer membrane to the periplasmic peptidoglycan network.
(taken from Alexander and Rietschel, 2001).

1.3.2. General structure of LPS

The historical discovery and elucidation of the general structure of LPS is a story which began in the late 19th century, following observations made by Pfeiffer in the laboratory of Robert Koch at the Institute for Infectious Diseases in Berlin. Pfeiffer discovered that lysates of heat-killed *Vibrio cholerae* could cause toxic shock reaction in guinea pigs, and postulated that this toxic substance was associated with the insoluble part of the bacterial cell wall; he named this substance endotoxin, from the Greek 'endo' meaning 'within' (Pfeiffer, 1892). The term 'lipopolysaccharide' has been adopted due to the later discovery that endotoxin consists of both lipid and carbohydrate (Erridge *et al.*, 2002). A recent review by Beutler and Rietschel (2003) provides a fascinating summation of the story of LPS discovery and its subsequent structural elucidation.

It is now known that all forms of LPS are comparable and conform to a common general structural architecture. Figure 2 provides a schematic diagram of this basic general structure, as described below. Variations of this basic arrangement account for the lipopolysaccharides of all Gram-negative bacteria so far investigated, and can be classified as containing three distinct regions: (i) lipid A, the highly hydrophobic and endotoxically active part of the molecule that is embedded in the outer membrane unless bacteria are lysed. (ii) core oligosaccharide, which is covalently attached to lipid A and can be further divided into inner and outer core. The inner core is proximal to lipid A, and contains a high proportion of unusual sugars such as 2-keto-3-deoxyoctulosonic acid (Kdo) and L-glycero-D-*manno*-heptose (Hep) (Alexander and Rietschel, 2001; Erridge *et al.*, 2002). The outer core extends further from the bacterial surface and mainly consists of more common hexose sugars such as glucose, galactose, *N*-acetyl glucosamine and *N*-acetyl galactosamine (Alexander and Rietschel, 2001; Erridge *et al.*, 2002). (iii) a serotype-specific polysaccharide (O-polysaccharide or O-specific chain) that extends outward from the bacterial surface, also typically composed of common hexoses.

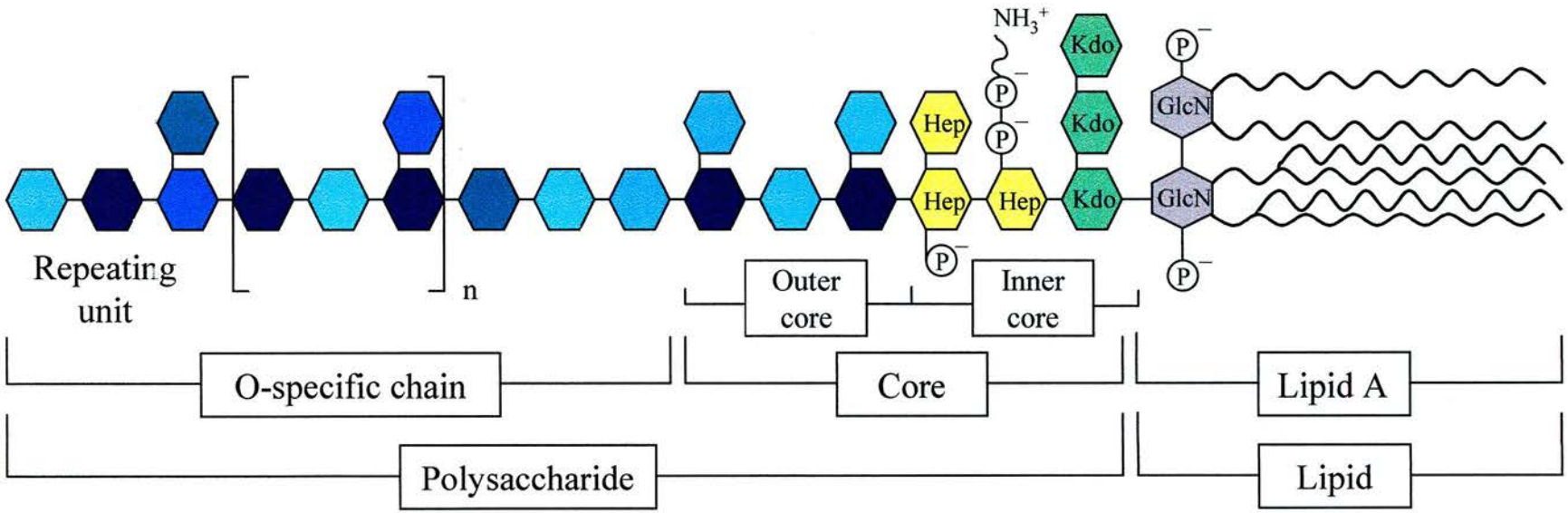


Figure 2. General structure of LPS from Gram-negative enterobacteria.

In the depicted general scheme of smooth-form LPS architecture, the terminal O-specific chain (O-polysaccharide) may be formed by up to 50 repeating units. In the core region, an inner and outer core can be distinguished on account of the different sugars present in these domains. The lipid A domain represents the primary immunostimulatory centre of LPS, determining endotoxicity in mammalian species. Long-chain fatty acids (acyl chains) are shown attached to the disaccharide of lipid A. Each individual hexagon represents a monosaccharide subunit. Abbreviations: Hep, D-glycero-D-*manno*-heptose; Kdo, 2-keto-3-deoxyoctulosonic acid; GlcN, glucosamine; P, phosphate; NH_3^+, ethanolamine. Adapted from Haeffner-Cavaillon *et al.* (1998) and Alexander and Rietschel (2001).

The O-polysaccharide is not ubiquitous as it can be truncated or absent in Gram-negative strains; for example, some bacterial strains may express LPS with a reduced non-repeating O-chain known as lipo-oligosaccharide (LOS) (Erridge *et al.*, 2002). Moreover, certain strains exhibit mutations in the '*rfb*' locus, which contains several genes involved in O-polysaccharide synthesis and attachment, and these strains are termed 'rough (R) mutants' to distinguish them from the wild-type 'smooth-form (S)' strains whose LPS contains O-polysaccharide (Erridge *et al.*, 2002).

1.3.2.1. O-polysaccharide

In LPS from most Gram-negative bacteria, the O-polysaccharide comprises up to 50 repeating oligosaccharide subunits, each formed with 2-8 monosaccharide moieties, in a highly species- and strain-specific manner (Alexander and Rietschel, 2001). The O-polysaccharides differ between strains by way of the individual sugars, sequence, chemical linkage, substitution, and ring forms utilised. This leads to an incredibly high structural diversity, even within a bacterial species, and therefore the necessity for classifying LPS by serotype according to the O-polysaccharide determinants. As the major Gram-negative bacterial antigen targeted by host antibody responses, the O-chain is often referred to as O-antigen and is a useful tool in typing strains and LPS (Erridge *et al.*, 2002).

As mentioned above, rough-mutant bacterial strains do not synthesise O-polysaccharide; as a consequence, these strains are less able to persist and survive *in vivo* since the O-chain protects bacteria from uptake by phagocytes, and prevents penetration of the complement membrane attack complex (Joiner *et al.*, 1984; Joiner *et al.*, 1986). However, many wild-type species of pathogenic Gram-negative bacteria have been identified which lack the O-polysaccharide moiety but can colonise mucosal surfaces *in vivo*, for example *Neisseria meningitidis*, *Haemophilus influenzae* and *Bordetella pertussis* (Peppler, 1984; Schneider *et al.*, 1984; Inzana *et al.*, 1985; Griffiss *et al.*, 1987). Interestingly, the LPS or LOS of organisms such as *N. meningitidis* and *H. influenzae* have also been found to contain terminal oligosaccharide structures that closely resemble human glycosphingolipids, due to

the presence of common host carbohydrates (Moran *et al.*, 1996). This mimicry of host glycoconjugates has been shown to contribute to increased resistance of the organism to phagocytosis and bactericidal activities (Verheul *et* al., 1993; Jarvis, 1995; Estabrook *et al.*, 1997). Moreover, in terms of resistance to host immunity, the variable O-polysaccharide domain provides a protective shield against access to the more conserved inner core and lipid A domains by antibacterial agents such as bile acids and cationic antimicrobial peptides (to be discussed in a later section), or by lipid A recognition proteins (Alexander and Rietschel, 2001).

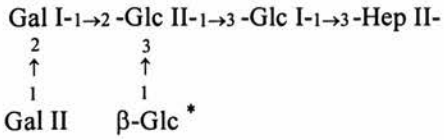
1.3.2.2. Core oligosaccharide

While the O-polysaccharide region is extremely variable, the oligosaccharide structures in the core domain are far more limited, with some regions being highly conserved between different strains and species. For example, and of relevance to this thesis which predominantly involves the use of LPS derived from the enterobacterium *Escherichia coli*, there are now over 160 identified *E. coli* O-serotypes but only 5 unique core structures (R1-R4 and K12; organisms containing these structures but no O-polysaccharide are therefore rough-form) (Amor *et al.*, 2000). These structures are presented diagrammatically in Figure 3A and will be discussed in more detail in Chapter 3 of this thesis, in which various *E. coli* LPS serotypes have been investigated experimentally.

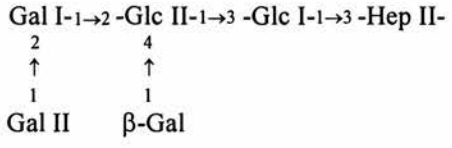
The outer core (also known as the 'hexose region') is generally more variable than the inner core. The inner core is characterised by more unusual sugars, particularly Kdo and heptose; Kdo has been demonstrated in virtually every identified LPS, and is α-bound to the carbohydrate backbone of lipid A. The only exceptions known are the LPS of *Acinetobacter* and *Burkholderia cepacia*, which exhibit 2-keto-D-glycero-D-talo-octonic acid (Ko) in its place (Erridge *et al.*, 2002). While the O-chain and majority of the core are dispensable in some viable mutants, Kdo is required for bacterial viability. The smallest saccharide moiety observed in the LPS of any organism is that of the deep rough mutant of *H. influenzae*, which has only one Kdo residue attached to lipid A (Helander *et al.*, 1988), while of the

A

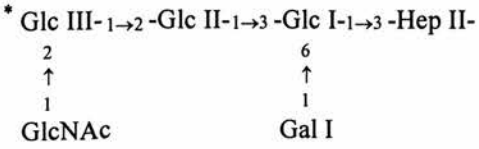
E. coli R1



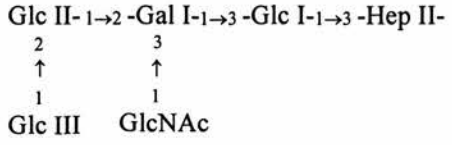
E. coli R4



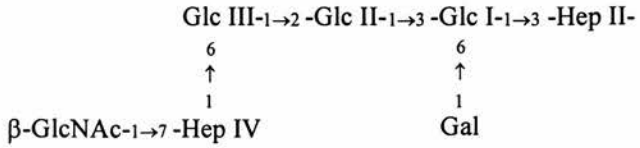
E. coli R2



E. coli R3



E. coli K12



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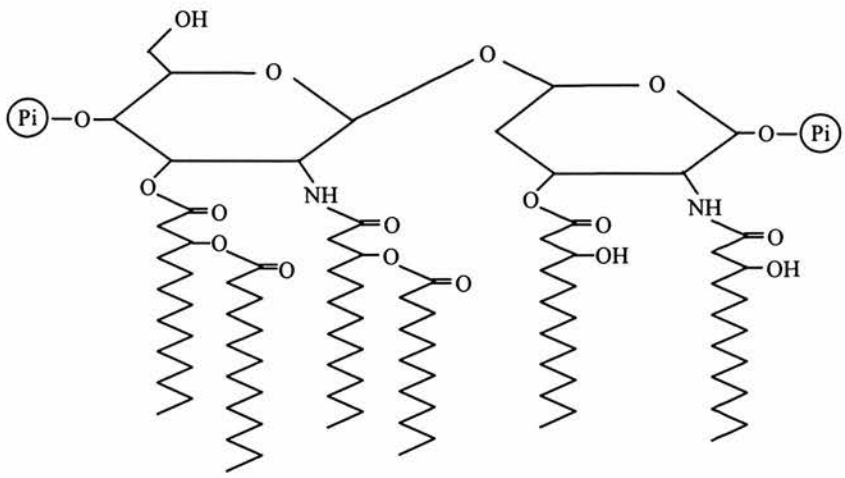


Figure 3. Structures of the five known outer core oligosaccharides from the LPS of *E. coli* (A), and the chemical structure of *E. coli* lipid A (B).

A. While there are over 160 identified *E. coli* O-serotypes, only 5 unique core structures have been determined. Although all 5 share a structural theme, with a tri-hexose carbohydrate backbone and two side chain residues, the order of hexoses in the backbone and the nature, position and linkage of the side chain residues can all vary. HepII is the last residue of the inner core oligosaccharide. * indicates the point of attachment of O-antigen, but this has only been determined experimentally for R1 and R2 (diagram adapted from Amor *et al.*, 2000). **B**. The structural format of *E. coli* lipid A is widely considered to be close to that optimally recognised by human cellular LPS receptors (bisphosphorylated diglucosamine backbone substituted with 6 acyl chains) (diagram adapted from Erridge *et al.*, 2002). Abbreviations: Glc, glucose; Hep, D-glycero-D-*manno*-heptose; Gal, galactose, GlcNAc, *N*-acetyl glucosamine; Pi, phosphate.

naturally occurring bacteria the smallest core seen is that of *Chlamydia* species, which consists of only a triplet of Kdo units (Brade *et al.*, 1987).

Both inner and outer core sugar residues may be substituted with anionic groups such as phosphate, diphosphate or diphosphoethanolamine (Holst *et al.*, 1996). In the inner core, these substituents associate closely with divalent cations such as Ca^{2+} and Mg^{2+}, which are required for membrane structure and function (Rietschel *et al.*, 1994). Interestingly, these cations may potentially be targeted for displacement by innate defence molecules such as cationic antimicrobial peptides, which can then interact electrostatically with the negative charges of the bacterial outer membrane prior to microbial killing (Koczulla and Bals, 2003).

1.3.2.3. Lipid A

Decades of research into the role played by lipid A in the endotoxicity of LPS have demonstrated that this domain harbours the endotoxic activity of the holo-molecule (reviewed by Alexander and Rietschel, 2001). Figure 3B provides a representation of the chemical structure of *E. coli* lipid A. Structurally, lipid A is typically composed of a β1,6-linked D-glucosamine (D-GlcN) disaccharide (β-D-GlcN-(1-6)-α-D-GlcN) carrying two phosphoryl groups at positions 1 and 4'; these phosphates can be further substituted with groups such as phosphate, ethanolamine, ethanolamine phosphate, ethanolamine diphosphate, GlcN, 4-amino-4-deoxy-L-arabinopyranose and D-arabino-furanose (Erridge *et al.*, 2002). To this structure are attached up to four acyl chains by ester or amide linkage, and these chains can in turn be substituted by further fatty acids to produce up to seven acyl substituents which may vary considerably between species in nature, number, length, order and saturation (Erridge *et al.*, 2002). These may be attached to lipid A either symmetrically (e.g. 3+3 as in *N. meningitidis* LPS) or asymmetrically (e.g. 4+2 as in *E. coli*) (Erridge *et al.*, 2002). The attachment site for the polysaccharide component of the molecule is invariably provided by a linking Kdo residue at position 6' (Beutler and Rietschel, 2003). A phosphorylated diglucosamine backbone attached to

at least one Kdo residue represents the most highly conserved region between LPS molecules (Erridge *et al.*, 2002).

Several studies have indicated that the major factors contributing to LPS endotoxicity are the number and lengths of acyl chains, and the phosphorylation state of the disaccharide backbone. Interestingly, a number of studies (reviewed by Erridge *et al.*, 2002) have additionally provided the conclusion that the optimal lipid A structure recognised by the host immune system to express maximal endotoxic responses is the structure exhibited by *E. coli*: a biphosphorylated hexa-acylated lipid A containing two β (1-6)-linked D-glucosamine residues, as shown by Figure 3B; deviations from this structure reduce the activity of the molecule (Rietschel *et al.*, 1994). However, it is important to note that the optimum structure for cellular activation appears to differ from that required for optimum cell binding: the criteria for cell binding are much less strict, requiring only a biphosphorylated disaccharide together with a minimum of two fatty acid substituents in no defined arrangement (Erridge *et al.*, 2002).

It is interesting to note that some lipid A structures can in fact exhibit pronounced inhibitory effects. For example, a structural variation known as compound 406, which is virtually identical to *E. coli* lipid A except it is tetra-acylated rather than hexa-acylated, completely lacks endotoxic activity and is also capable of inhibiting normal lipid A signalling in human cells (Wang *et al.*, 1990; Wang *et al.*, 1991; Wang *et al.*, 1992). It has further been suggested that *E. coli* lipid A may be detoxified by macrophages by a pathway involving biodegradation to a product resembling the endotoxically-inactive compound 406 (Erridge *et al.*, 2002).

Having described the prominent structural features of LPS and the roles they play in the architecture and activity of the molecule, the following section will describe the biological effects of LPS and its powerful immuno-stimulatory activities.

1.3.3. Activation of innate immunity by LPS

LPS is capable of inducing a broad spectrum of biological effects in the mammalian host. The primary target cells of LPS in mammalian species are the professional phagocytes of innate immunity (peripheral monocytes, tissue macrophages and neutrophils) which constitutively express the membrane-bound form of CD14 as well as TLR4 (these molecules are discussed in depth in section 1.4.) (Alexander and Rietschel, 2001). Dendritic cells have also been identified as a myeloid cell type that is strongly activated by LPS, in response to which these cells mature to play a key role as primary antigen-presenting cells (Cella *et al.*, 1997; Verhasselt *et al.*, 1997). LPS is also known to induce specific and rapid activation of humoral serine protease cascades such as the mammalian complement system (Galanos *et al.*, 1971; Cooper and Morrison, 1978). Moreover, LPS can directly or indirectly (via induction of early inflammatory cytokines) upregulate the production of local epithelial antimicrobial molecules such as human β-defensin-2 (HBD-2) and the low molecular weight proteinase inhibitors elafin and secretory leukocyte proteinase inhibitor (SLPI) (Sallenave *et al.*, 1994; Jin *et al.*, 1998; Becker *et al.*, 2000; Harder *et al.*, 2000). These antimicrobial molecules will be discussed in later sections.

Mononuclear cells react with tremendous sensitivity to the presence of LPS and secrete a range of endogenous mediators, including the pro-inflammatory cytokines TNF-α, macrophage migration inhibitory factor (MIF), interleukins IL-1β, IL-6, IL-8, IL-12, IL-15, and IL-18, the colony-stimulating factors G-CSF, M-CSF and GM-CSF, lipid-derived mediators such as platelet-activating factor (PAF), prostaglandin E_2 (PGE_2), thromboxane A_2 (TXA_2), leukotrienes, and reduced oxygen species such as the superoxide anion (O_2^-), hydroxyl radicals (OH) and nitric oxide (NO); Figure 4 provides a diagrammatic representation of these pleiotropic effects of LPS-mediated monocyte/macrophage activation (Alexander and Rietschel, 2001).

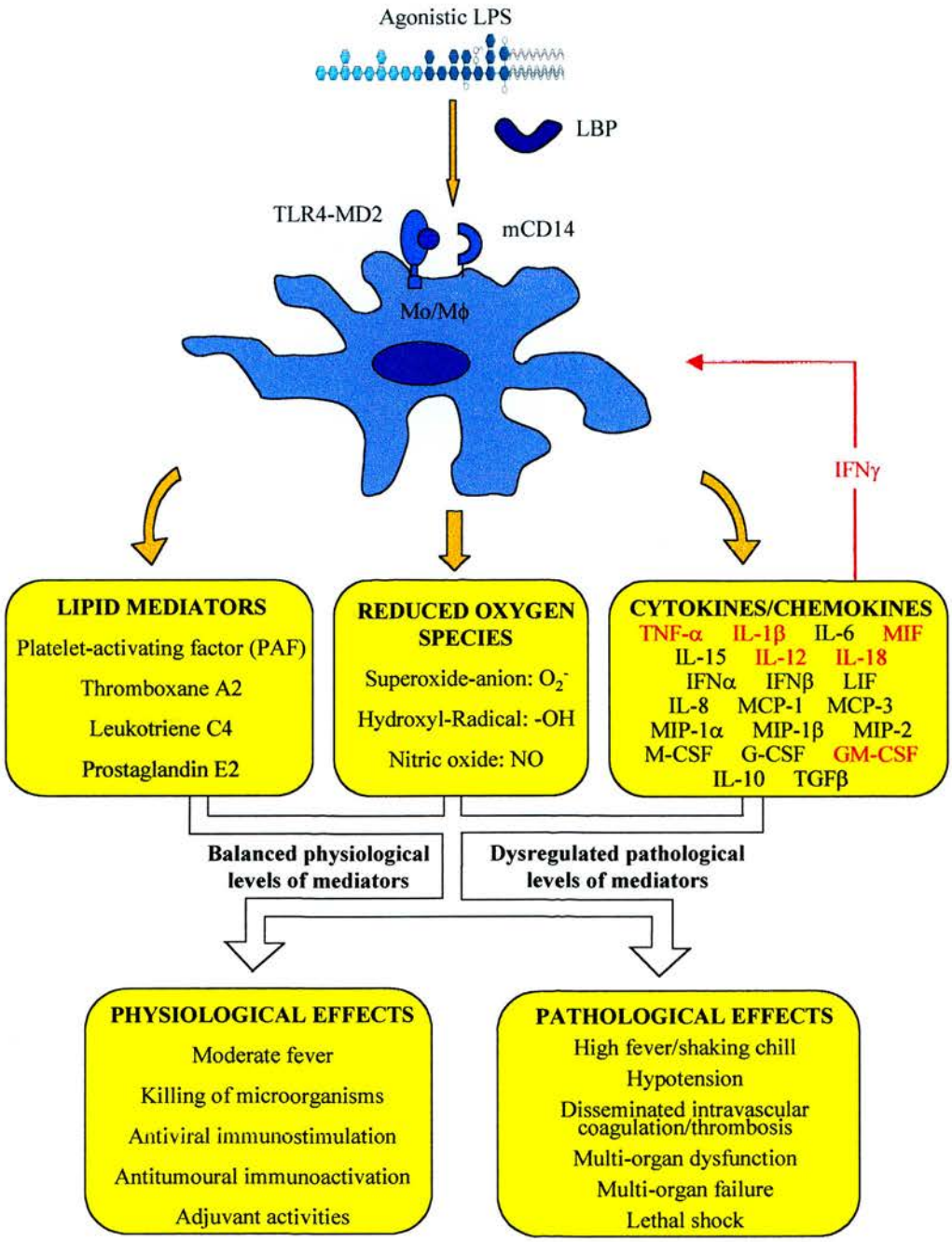


Figure 4. The pleiotropic effects of LPS-mediated macrophage activation

This diagram depicts the central extracellular route for LPS-mediated activation of peripheral monocytes or tissue macrophages (see section 1.4.), and the major inflammatory spectrum of cellular responses. In normal physiological conditions, reasonable and balanced concentrations of the above mediators lead to activation of general antimicrobial, antiviral and antitumoural defence mechanisms, but during pathological situations dysregulated and unbalanced release of mediators may lead to life-threatening effects such as those observed in severe forms of sepsis. Key mediators which either reinforce or suppress the pro-inflammatory activity of monocytes/macrophages are highlighted in red and blue, respectively. In T-lymphocytes, natural killer (NK) cells and other cell types, IL-12 and IL-18 induce release of IFN-γ, which hypersensitises mononuclear phagocytes to LPS. Diagram is adapted from Alexander and Rietschel (2001).

1.3.3.1. Severe sepsis and septic shock

While the response to the presence of Gram-negative bacteria and/or LPS can be of benefit to the host by activating mechanisms aimed at eradicating the invading pathogen (such as phagocytosis of bacteria, activation of the classical complement pathway by lipid A and the alternate or lectin pathway by polysaccharide, or release of antimicrobial peptides), an over-zealous response can lead to overt inflammation, tissue damage and possibly even death (see Figure 4). The most extreme consequence of an overtly vigorous and uncontrolled response to the presence of Gram-negative bacteria and LPS is severe sepsis leading to shock; this condition is the principal cause of death in non-cardiac intensive care units (ICUs) (Lolis and Bucala, 2003). Sepsis can be defined as 'severe' when organ dysfunction develops, such as acute renal failure, and 'septic shock' describes severe sepsis associated with hypotension that is unresponsive to fluid resuscitation (Lolis and Bucala, 2003). Septic diseases affect more than 50% of all patients in ICUs (Brun-Buisson, 2000). It is important to note that Gram-negative bacteria currently account for roughly half of the reported cases of sepsis, while Gram-positive bacteria and fungal organisms are contributing to a progressively higher proportion (Martin *et al.*, 2003).

Particularly high risks for septic syndromes are patients in ICUs suffering from polytrauma, burn injuries or following major surgery and organ transplantation under high-dose immunosuppression (Alexander and Rietschel, 2001). In healthy humans, the maintenance of low systemic levels of LPS is normally guaranteed by the effective external barriers of innate immunity, and an efficient physiological clearance system for the small dose of gastrointestinal LPS that constitutively enters the circulation with nutritional lipids (Jacob *et al.*, 1977). Accordingly, most cases of Gram-negative sepsis are caused by the enterobacteria *E. coli* and *Klebsiella* species (Bochud and Calandra, 2003). Normal concentrations of LPS detectable in human peripheral blood are around 3-10pg/ml, while levels higher than 300pg/ml are characteristic during severe sepsis or septic shock (Casey *et al.*, 1993; Opal *et al.*, 1999).

Whilst cytokines such as MIF (for which a role in the pathogenesis of sepsis has been suggested by studies demonstrating that MIF-deficient mice are highly resistant to endotoxaemia (Bozza *et al.*, 1999)) may be important mediators during sepsis, TNF-α and IL-1β are the prototypic inflammatory cytokines that mediate many of the immunopathological features of LPS-induced shock (Dinarello, 1997). They are released during the first 30-90 minutes following LPS exposure and in turn activate a second level of inflammatory cascades including cytokines, lipid mediators and reactive oxygen species, in addition to upregulating cell adhesion molecules that result in initiation of inflammatory cell migration into tissues (Cohen, 2002). TNF-α in particular appears to be a key mediator in endotoxaemia, since passive immunisation against this cytokine was shown to substantially attenuate the lethal effects of LPS in mice (Beutler *et al.*, 1985), and the effects of LPS could be mimicked by administration of recombinant TNF-α to animals in high doses (Tracey *et al.*, 1986). Figure 5 describes the biological activities and cell and tissue targets of TNF-α, relevant to its role as a mediator of sepsis. It is important to note that, while TNF-α may instigate a range of potentially damaging inflammatory sequelae, a number of studies using TNF-α deficient mice have demonstrated that it also plays a crucial role in defence against microbial pathogens (reviewed by Mannel and Echtenacher, 2000). However, with regards to the pathogenesis of sepsis, strategies to downregulate the pro-inflammatory effects of TNF-α may be beneficial.

Much work is currently concerned with uncovering novel strategies for the treatment of sepsis, including the potential use of antimicrobial peptides that display anti-endotoxin activity (Koczulla and Bals, 2003). Cationic antimicrobial peptides will be discussed in a later section. However, other approaches currently under investigation for potential use in the treatment of sepsis will not be discussed here; several authors have provided recent detailed reviews of past and ongoing studies into such strategies, to which the reader is referred (Bochud and Calandra, 2003; Hotchkiss and Karl, 2003; Lolis and Bucala, 2003; Marshall, 2003; Riedemann *et al.*, 2003). Suffice to say that the discovery of novel approaches aimed at dampening the systemic inflammatory response to Gram-negative organisms and LPS is a desirable goal for researchers.

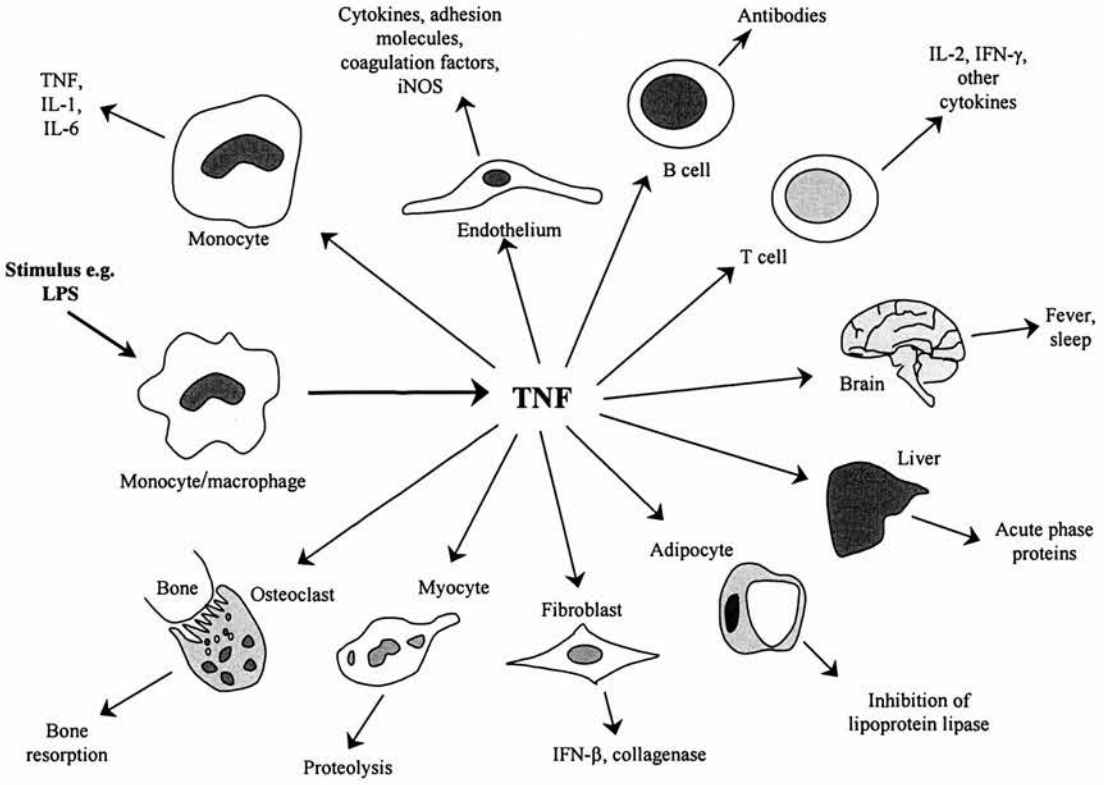


Figure 5. Biological activities of tumour necrosis factor (TNF)-α

Although TNF-α is produced by several cell types, including neutrophils, T cells and mast cells, the main source of this cytokine is monocytes/macrophages. TNF-α induces a range of pro-inflammatory changes in endothelial cells, including cytokine production, adhesion molecule expression, release of pro-coagulatory substances and induction of inducible nitric oxide synthase (iNOS). These alterations may ultimately lead to septic shock. TNF-α also stimulates T and B cells, induces fever in the brain, suppresses lipoprotein lipase in adipocytes (contributing to cachexia or tissue wasting, hence the alternative name for TNF-α, 'cachectin') and stimulates hepatocytes to produce acute phase proteins. Fibroblasts and osteoclasts are target cells for TNF-α in rheumatoid arthritis. Diagram is adapted from Eigler *et al.* (1997).

Having examined the structural features and biological activities of LPS, the following sections will discuss the current knowledge surrounding the mechanisms by which the innate immune system recognises and responds to LPS. In this regard, the Toll-like receptors are now known to play a major role in LPS recognition and signalling.

1.4. LPS RECOGNITION AND SIGNALLING PATHWAYS

1.4.1. TOLL-LIKE RECEPTORS

1.4.1.1. Overview

Toll-like receptors comprise a family of type I transmembrane proteins that are evolutionarily conserved between insects and humans (Medzhitov *et al.*, 1997; Rock *et al.*, 1998). Toll was first identified as an essential molecule for embryonic dorsoventral patterning in the *Drosophila* fruitfly (Hashimoto *et al.*, 1988), and was subsequently shown to be important for *Drosophila* immunity via induction of the antifungal peptide Drosomycin (Lemaitre *et al.*, 1996). The discovery of a mammalian homologue of the *Drosophila* Toll by Medzhitov *et al.* (1997), now designated TLR4, has led to a rapid increase in the knowledge surrounding the roles of these receptors in mammalian immunity.

TLRs are characterised by an amino-terminal extracellular leucine-rich repeat (LRR) domain, and a carboxy-terminal intracellular tail containing a conserved region known as the Toll/interleukin-1 (IL-1) receptor (TIR) domain (Rock *et al.*, 1998). The extracellular LRR domain of TLRs is involved in ligand-binding, and may also be necessary for TLR dimerisation (Singh *et al.*, 2003); LRRs are found in a diverse set of proteins in which they are involved in ligand recognition and signal transduction (Kobe and Deisenhofer, 1995). While the extracellular LRR domain of TLRs displays a high degree of polymorphism in order to confer ligand specificity, the intracellular TIR domain is a conserved module which mediates protein-protein interactions between the TLRs and signal-transduction components; thus the TIR domain promotes intracellular signalling and transcription of genes involved in

immune activation (Takeda *et al.*, 2003). The TIR domain is also present in the cytoplasmic portions of members of the IL-1 receptor (IL-1R) family, including IL-1R and IL-18R (Medzhitov, 2001). In mammals, the TIR domain can also be found in several cytoplasmic proteins, including two signalling adapters known as MyD88 (myeloid differentiation factor 88) (Medzhitov *et al.*, 1998) and TIRAP (TIR domain-containing adapter protein) (Horng *et al.*, 2001); both of these molecules function in TLR signal transduction and will be discussed in a later section.

To date, the mammalian TLR family is known to consist of ten members (TLR1-TLR10) (Takeda *et al.*, 2003). Each of these TLRs seems to have a distinct function in innate immune recognition of microbial components, and ligands have been identified for most of these receptors (Table 2). It is evident that the TLRs are responsible for modulation of immune responses to a variety of stimuli, although the overall innate immune response to infection is likely to represent the sum of signals generated by the interaction of multiple TLRs.

Of particular relevance to this study, the first- and best-characterised member of the TLR family, TLR4, has been identified as the crucial receptor for recognition of LPS. The following sections will discuss the role of TLR4 in regulation of LPS responses.

1.4.1.2. Toll-like receptor 4 (TLR4) and LPS recognition

As mentioned previously, TLR4 was the first characterised mammalian Toll (Medzhitov *et al.*, 1997). The study by Medzhitov *et al.* (1997) demonstrated that a dominant active form of this receptor induced cytokine expression and NF-κB activation when expressed in monocytic cell lines. Since that seminal study, TLR4 has been shown to function as the signal-transducing receptor for LPS.

TLR family	Ligands (origin)
TLR1	Tri-acyl lipopetides (bacteria, mycobacteria) Soluble factors (*Neisseria meningitidis*)
TLR2	Lipoprotein/lipopeptides (variety of pathogens) Peptidoglycan (Gram-positive/-negative bacteria) Lipoteichoic acid (Gram-positive bacteria) Lipoarabinomannan (mycobacteria) Phenol-soluble modulin (*Staphylococcus epidermidis*) Glycoinositolphospholipids (*Trypanosoma cruzi*) Glycolipids (*Treponema maltophilum*) Porins *(Neisseria*) Zymosan (fungi) Atypical LPS (*Leptospira interrogans*) Atypical LPS (*Porphyromonas gingivalis*) HSP70 (host)
TLR3	Double-stranded RNA (virus)
TLR4	LPS (Gram-negative bacteria) Taxol (plant) Fusion protein (respiratory syncytial virus) Envelope proteins (mouse mammary tumour virus) HSP60 (*Chlamydia pneumoniae*) HSP60 (host) HSP70 (host) Type III repeat extra domain A of fibronectin (host) Oligosaccharides of hyaluronic acid (host) Polysaccharide fragments of heparan sulphate (host) Fibrinogen (host)
TLR5	Flagellin (bacteria)
TLR6	Di-acyl lipopeptides (mycoplasma)
TLR7	Imidazoquinoline (synthetic compound) Loxoribine (synthetic compound) Bropirimine (synthetic compound)
TLR8	?
TLR9	CpG DNA (bacteria)
TLR10	?

Table 2. Toll-like receptors and their ligands
(adapted from Takeda *et al.* (2003))

This discovery was made as a result of the work carried out by two independent groups, who strove to identify the genes responsible for the LPS-hyporesponsive phenotype of the classical mouse strains C3H/HeJ and C57BL/10ScCr. These studies found that the defective *Lps* gene, associated with profound LPS-hyporesponsiveness in these strains, was in fact identical to the *TLR4* gene (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999). The C3H/HeJ mouse strain has a point mutation in the intracellular TIR domain of TLR4, leading to a dominant-negative mutant containing a proline to histidine substitution in position 712 (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999). The mutant TLR4 from C3H/HeJ mice has since been shown to be defective in binding to the adapter protein MyD88, thus failing to activate central downstream signalling pathways (Rhee and Hwang, 2000). Transfection of this missense mutant *Tlr4* into the RAW 264.7 murine macrophage cell line was shown to inhibit LPS-induced NF-κB activation (Rhee and Hwang, 2000). The C57BL/10ScCr mouse strain was found to harbour a homozygous null mutation in the *Tlr4* gene (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999) correlating with a deletion in the third exon (Poltorak *et al.*, 2000). TLR4-deficient mice generated by gene targeting were also hypo-responsive to LPS, confirming the role for TLR4 in mediating inflammatory signals from LPS (Hoshino *et al.*, 1999). Moreover, TLR4 mutations have also been found to be associated with endotoxin hyporesponsiveness in humans (Arbour *et al.*, 2000; Schwartz, 2001).

TLR4 is expressed predominantly in immune cells of the myeloid lineage, such as dendritic cells (DCs) and monocytes/macrophages (Akashi *et al.*, 2000; Muzio *et al.*, 2000; Kadowaki *et al*, 2001; Visintin *et al.*, 2001b). Mast cells, which play a role in phagocytosing pathogens, processing antigens and releasing inflammatory cytokines (Malaviya and Abraham, 2001), also recognise Gram-negative bacteria via TLR4 (McCurdy *et* al., 2001; Supajatura *et al.*, 2001). However, TLR4 expression has been observed in most human tissues (Zarember and Godowski, 2002) and, in addition to professional innate immune cells, TLR4 is expressed in several other cells types that contribute to inflammatory responses. For example, TLR4 is found in endothelial cells (Faure *et* al., 2000; Zeuke *et al.*, 2002), fibroblasts (Wang and Ohura, 2002), adipocytes (Lin *et al.*, 2000) and in cells of

epithelial origin, such as those of the pulmonary (Monick *et al.*, 2003; Guillot *et al.*, 2004), intestinal (Cario *et al.*, 2000; Hornef *et al.*, 2002; Hornef *et al.*, 2003), renal (Wolfs *et al.*, 2002), bladder (Backhed *et al.*, 2002), colonic and gingival epithelia (Uehara *et al.*, 2001).

Recent studies have provided interesting findings with regards to the expression and localisation of TLR4 in both intestinal and pulmonary epithelial cells. These cells are constantly exposed to bacterial-related antigens, whether pathogenic or commensal, and yet in normal circumstances exhibit only minimal immunological responses (Boman, 2000). Several studies have documented very low or absent surface expression of TLR4 in intestinal epithelial cells (Abreu *et al.*, 2001; Naik *et al.*, 2001), and also lack of production of CD14 (Cario *et al.*, 2000) and the adapter protein MyD88 (Abreu *et al.*, 2001); Abreu *et al.* (2001) further demonstrated that LPS responsiveness could be enhanced by co-transfection of TLR4-MD-2 (myeloid differentiation protein-2, an accessory molecule for TLR4; see following section). While surface expression of TLR4 is relatively low in normal intestinal epithelium, Cario and Podolsky (2000) found that TLR4 expression is augmented in inflammatory bowel diseases such as Crohn's disease and ulcerative colitis, providing a rationale for the exaggerated inflammatory responses to bacterial flora in these conditions. Similarly, pulmonary epithelial cells of alveolar and tracheobronchial origin have been shown to lack surface expression of TLR4 under normal conditions (Monick *et al.*, 2003; Tsutsumi-Ishii, 2003). The relative tolerance of normal airway epithelial cells to foreign antigens is altered in individuals with asthma and following respiratory syncytial virus (RSV) infection, and subsequent development of asthma has been linked to severe infection with RSV during the first year of life (Stein *et al.*, 1999; Welliver, 1999); the study by Monick *et al.* (2003) provided a possible basis for this observation, by demonstrating that RSV up-regulates TLR4 expression and membrane localisation in airway epithelial cells, and consequently sensitises the airway epithelium to LPS-mediated inflammation.

While observations regarding surface expression of TLR4 in these epithelial cells have been relatively discordant, recent studies have further demonstrated that

TLR4 resides intracellularly in the Golgi apparatus in intestinal epithelial cells (Hornef *et al.*, 2002; Hornef *et al.*, 2003). These studies suggest that, in contrast to the situation in macrophages, LPS recognition in intestinal epithelial cells relies upon co-localisation of TLR4 and LPS in Golgi apparatus following LPS internalisation (Hornef *et al.*, 2002; Hornef *et al.*, 2003); previous work has demonstrated delivery of LPS to the Golgi apparatus following internalisation (Thieblemont and Wright, 1999). A role for intracellular TLR4 in LPS recognition in intestinal epithelial cells was supported by the observation that monoclonal antibodies directed against the TLR4-MD-2 complex failed to block TLR4-mediated signalling (Hornef *et al.*, 2002; Suzuki *et al.*, 2003), while the same strategy was successful in neutralising LPS response in macrophages (Schimazu *et al.*, 1999; Akashi *et al.*, 2000). Similarly, a recent study by Guillot *et al.* (2004) demonstrated that TLR4 was not detectable on the surface of pulmonary epithelial cells, but was constitutively expressed and localised intracellularly; these findings were again supported by the inability of an anti-TLR4 monoclonal antibody to inhibit cellular activation by LPS (Guillot *et al.*, 2004).

Interestingly, the potently pro-inflammatory cytokine interferon-γ (IFN-γ), which has long been known to play a role in priming cells for inflammatory responses to PAMPs such as LPS, has recently been shown to augment the LPS activation of cell types such as monocytes/macrophages, intestinal epithelial cells and renal epithelial cells by enhancing the expression of TLR4 and MD-2 (Bosisio *et al.*, 2002; Wolfs *et al.*, 2002; Suzuki *et al.*, 2003).

1.4.1.3. The LPS receptor complex

In addition to TLR4, recognition of LPS requires the cooperative involvement of a number of molecules known collectively as the 'LPS receptor complex' (Figure 6) (reviewed by Fujihara *et al.*, 2003).

LPS is firstly bound by the 60 kilodalton (kDa) acute phase serum glycoprotein LPS-binding protein (LBP), via specific recognition of the lipid A

domain of LPS (Tobias *et al.*, 1986; Tobias *et al.*, 1989; Schumann *et al.*, 1990). LBP functions to extract single LPS molecules from aggregated structures and catalyse the transfer of these monomers to CD14, a 55kDa glycoprotein present either as a glycosylphosphatidylinositol (GPI)-anchored form on the surface of myeloid cells (mCD14), or circulating in serum as a soluble form lacking the GPI tail (sCD14) (Haziot *et al.*, 1988; Wright *et al.*, 1990; Hailman *et al.*, 1994; Tobias *et al.*, 1995; Hailman *et al.*, 1996). Besides LPS, CD14 is also required for the recognition of other bacterial products including peptidoglycan, lipoteichoic acid and lipoarabinomannan (Pugin *et al.*, 1994; Gupta *et al.*, 1996; Savedra *et al.*, 1996).). In addition to their critical roles in provision of monomerised LPS to the surface of phagocytes for initiation of signal activation, both LBP and m/sCD14 have been shown to mediate rapid cellular internalisation of LPS aggregates and phagocytosis of intact Gram-negative bacteria (Gegner *et al.*, 1995; Schiff *et al.*, 1997; Kitchens and Munford, 1998; Thieblemont and Wright, 1999; Vasselon *et al.*, 1999). Within the group of professional human phagocytes, CD14 is constitutively expressed at high levels by monocytes and tissue macrophages (approximately 10^5 molecules/cell), and to a much lower extent by neutrophils (approximately 3×10^3 molecules/cell) (Antal-Szalmas *et al.*, 1997).

The important catalytic role of LBP in LPS-induced cell activation has been underlined by the observation that blood from mice with a targeted deletion of the LBP gene was hyporesponsive to LPS by at least 1000-fold (Wurfel *et al.*, 1997). Similarly, CD14-deficient mice are highly resistant to endotoxic shock, and monocytes derived from CD14-deficient mice are hyporesponsive to LPS (Haziot *et al.*, 1996). Soluble CD14 accepts LPS from LPS/LBP complexes and facilitates LPS-dependent activation of CD14-negative cells, such as endothelial and epithelial cells (Pugin *et al.*, 1993; Backhed *et al.*, 2002); interestingly, several studies have recently demonstrated CD14 on the surface membrane of intestinal and airway epithelial cells (Becker *et al.*, 2000; Hornef *et al.*, 2002; Tsutsumi-Ishii and Nagaoka, 2003), and therefore the roles of mCD14/sCD14 in LPS-mediated activation of these cells currently requires further definition. Furthermore, work has shown that LBP is

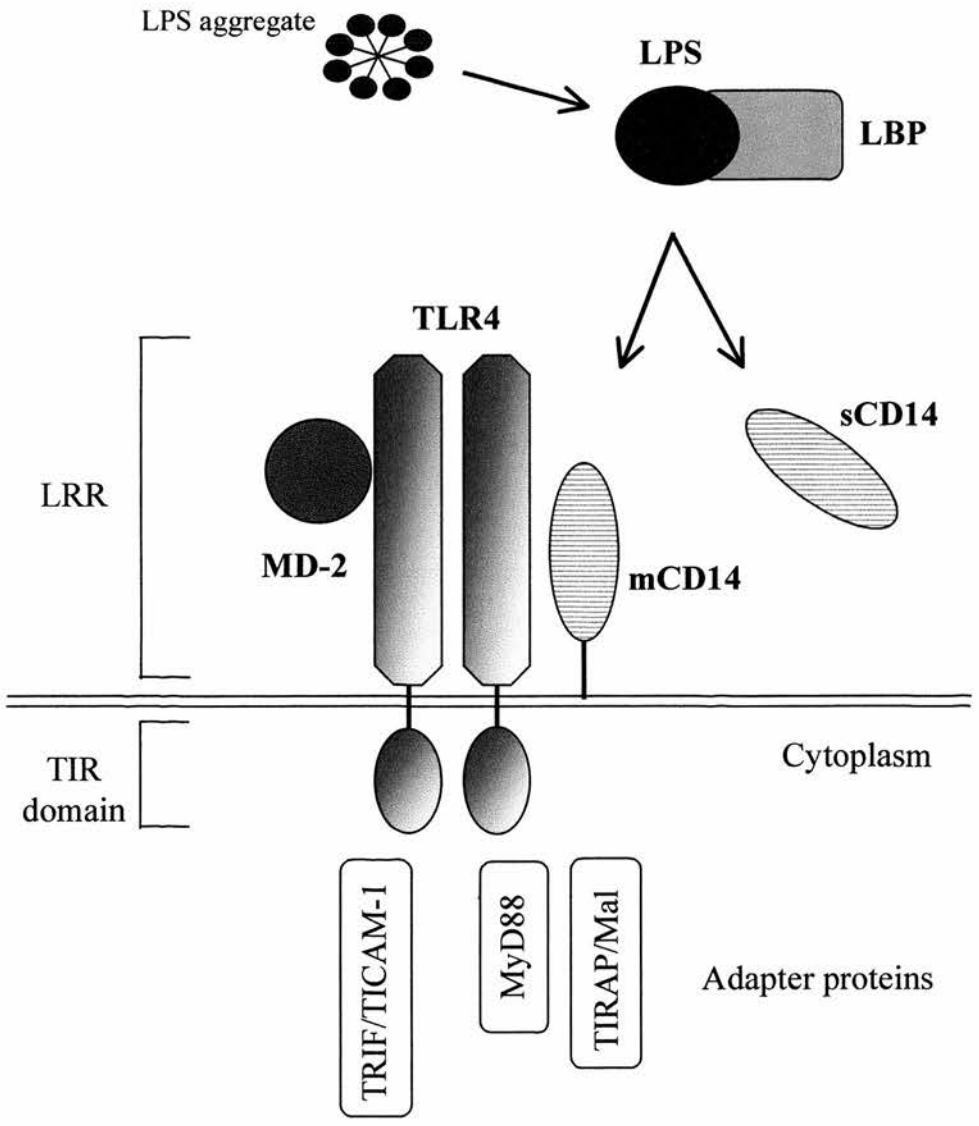


Figure 6. Schematic diagram of the LPS receptor complex.

LPS is recognised by a complex of three proteins: CD14, TLR4 and MD-2. The serum glycoprotein LPS-binding protein (LBP) disaggregates LPS and transfers it to CD14, which exists in two forms: mCD14, anchored on the surface of myeloid cells such as monocytes/macrophages, and sCD14 which circulates in plasma. The LPS/sCD14 complex can activate cells that lack mCD14, such as endothelial and epithelial cells. CD14 presents LPS to TLR4-MD-2. MD-2 plays a role in LPS recognition and regulates cellular distribution of TLR4. TLR4 is a transmembrane protein characterised by an extracellular domain containing multiple leucine-rich repeats (LRRs), a transmembrane domain, and an intracellular TIR domain. TLR4 also plays a role in LPS recognition and functions as the signal-transducing receptor for LPS. Three adapter proteins are responsible for TLR4-mediated signalling: MyD88, TIRAP (also known as Mal) and TRIF (also known as TICAM-1).

produced not only by liver cells but also locally by epithelial cells in the lung (Dentener *et al.*, 2000).

LPS stimulation is followed by increased physical proximity between CD14 and TLR4, suggesting that CD14 and TLR4 may interact in order to activate LPS signalling (Jiang *et al.*, 2000; Correia *et al.*, 2001). An interesting observation to note at this point is that, while it is now recognised that TLR4 represents the principal LPS signal-transducing molecule, atypical LPS such as that exhibited by *Porphyromonas gingivalis* is recognised by TLR2 (Hirschfeld *et al.*, 2001). Conformational differences in the shape of the lipid A portion in these LPS types have recently been suggested to provide the basis for this disparity: if the lipid A portion is conical in shape (as in *E. coli* LPS) it will be recognised by TLR4, whereas if it is cylindrical (as in *P. gingivalis* LPS) it will be recognised by TLR2 (Netea *et al.*, 2002).

TLR4 is essential for LPS signalling; however, overexpression of TLR4 alone does not confer LPS responsiveness on LPS-unresponsive cell lines such as HEK (human embryonic kidney) 293 cells (Kirschning *et al.*, 1998). MD-2, a small (18kDa) protein that lacks a transmembrane domain, was identified as a molecule that associates with the extracellular portion of TLR4 and enhances LPS responsiveness (Shimazu *et al.*, 1999; Akashi *et al.*, 2000). Notably, Chinese hamster ovary (CHO) cell lines that are hyporesponsive to LPS have mutations in the *MD-2* gene, and this phenotype could be reversed on transfection of MD-2 cDNA or by addition of soluble MD-2 (Schromm *et al.*, 2001). Moreover, as is the case for TLR4-deficient mice, MD-2-deficient mice are resistant to LPS-induced shock; macrophages, dendritic cells and B cells from these mice also display severely impaired responses to LPS (Nagai *et al.*, 2002). However, MD-2 (-/-) mice were found to be susceptible to *Salmonella typhimurium* infection (Nagai *et al.*, 2002). MD-2 associates with TLR4 in the endoplasmic reticulum/Golgi apparatus before the TLR4/MD-2 complex moves to the cell surface, where excess MD-2 is secreted (Visintin *et al.*, 2001a). MD-2 has additionally been shown to play an essential role in the distribution of TLR4 since, whereas in wild-type cells TLR4 is normally

located on the cell surface, it was found in the Golgi apparatus in cells lacking MD-2 (Nagai *et al.*, 2002).

1.4.1.4. Myeloid-differentiation factor (MyD88)-dependent TLR4 signalling

Since the TLRs share sequence homology with the IL-1 receptor (IL-1R) in their cytoplasmic 'TIR' domains, it is considered that TLRs and the IL-1R use homologous signalling pathways. The downstream events are mediated by common components: the adapter protein MyD88, a family of IL-1 receptor-associated kinases (IRAK) and another adapter protein, TNF receptor-associated factor 6 (TRAF6). MyD88, which contains an N-terminal death domain and a C-terminal TIR domain, is recruited to TLR4 following ligation of TLR4 with LPS. The TIR domain of MyD88 then associates with the TIR domain of TLR4, whereas the death domain interacts with the N-terminal death domain of the serine/threonine kinase IRAK and recruits IRAK to the receptor complex (Muzio *et al.*, 1997; Wesche *et al.*, 1997; Burns *et al.*, 1998; Medzhitov *et al.*, 1998) (the IRAK family is reviewed by Fujihara *et al.*, 2003). IRAKs are subsequently phosphorylated, become dissociated from the receptor complex and interact with TRAF6 (Cao *et al.*, 1996; Muzio *et al.*, 1998). TRAF6 activates mitogen-activated protein kinases (MAPK), and also the inhibitors of κB (IκB) kinase (IKK) complex. The IKK complex is composed of two catalytic subunits, IKKα and IKKβ, and one regulatory subunit, IKKγ (Takeda *et al.*, 2003). This complex induces phosphorylation of the inhibitory protein IκB, which functions to sequester NF-κB in the cytoplasm; phosphorylation is followed by polyubiquitination of the IκBs and their 26S proteasome/multicatalytic proteinase complex (MPC) -mediated degradation, allowing NF-κB to translocate to the nucleus and activate transcription of a multitude of genes, including TNF-α and IκBα (reviewed by Baldwin, 1996, and Hilt and Wolf, 1996). The latter provides a negative feedback loop whereby newly synthesised IκB binds to NF-κB and terminates its activity (Silverman and Maniatis, 2001). Figure 7 provides a schematic diagram of the TLR4 signalling pathway activated by LPS, as described in this section and the section below (section 1.4.1.5). This diagram also includes proposed mechanisms of TLR4-independent LPS recognition, as described in section 1.4.2.

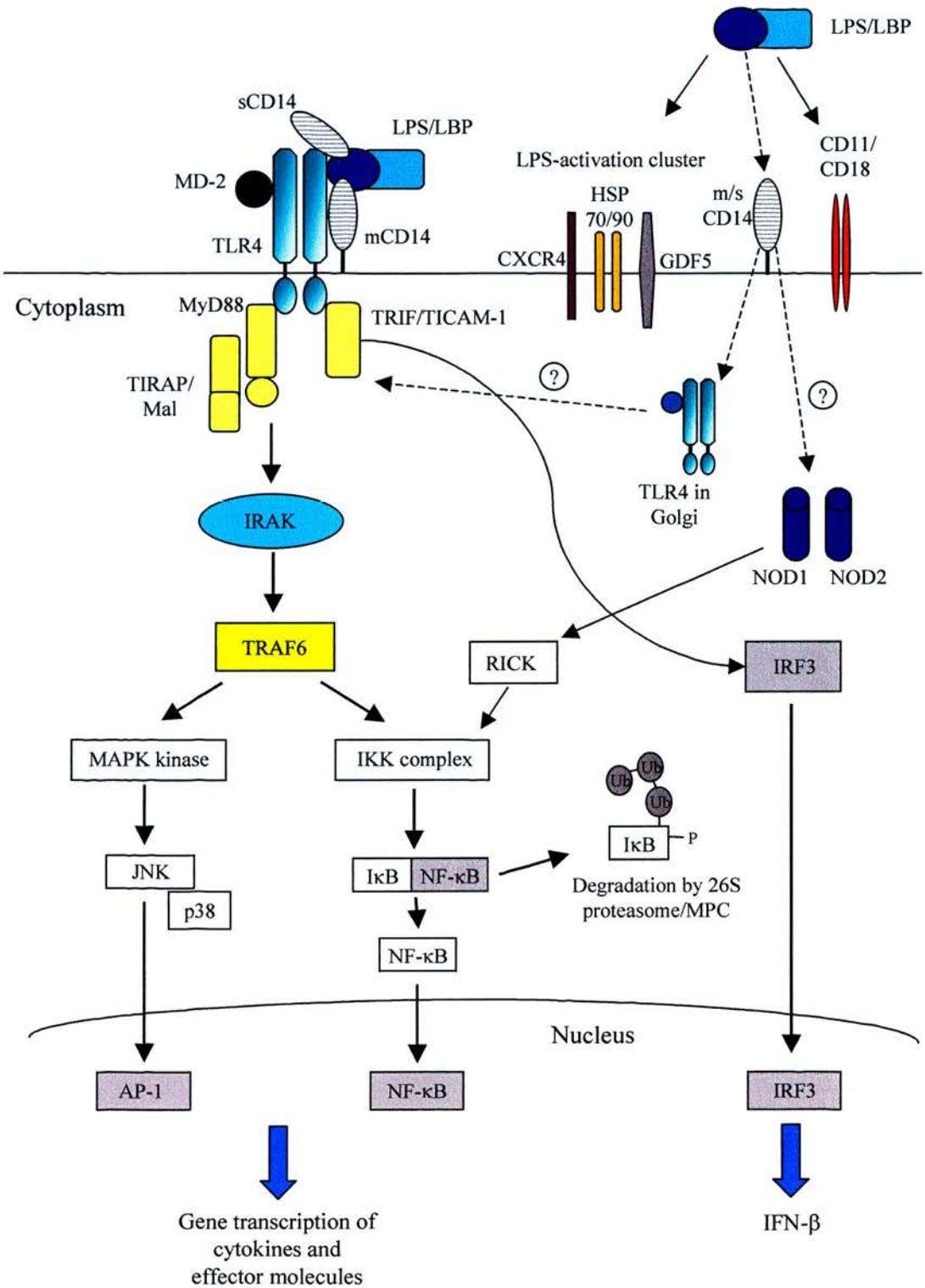


Figure 7. Schematic diagram of TLR4-dependent LPS recognition and signalling, and proposed mechanisms of TLR4-independent LPS recognition.

LPS activates inflammatory gene transcription via signalling pathways downstream of TLR4. TLR4-independent mechanisms of LPS recognition have also been proposed. These pathways are described in detail in sections 1.4.1.4, 1.4.1.5 and 1.4.2.

The involvement of signalling molecules such as MyD88, IRAK-1, IRAK-2 and TRAF6 in the LPS-mediated activation of NF-κB was confirmed in a study which demonstrated that the dominant-negative construct of each molecule could block LPS-induced activation of NF-κB in human monocytes and endothelial cells (Zhang *et al.*, 1999). Moreover, MyD88-, IRAK-1- and TRAF6-deficient mice are hyporesponsive to LPS (Kawai *et al.*, 1999; Lomaga *et al.*, 1999; Swantek *et al.*, 2000). Macrophages of MyD88-deficient mice were also shown to be unable to activate IRAK-1, and did not produce the inflammatory cytokines TNF-α, IL-6 and IL-1β on stimulation with LPS (Kawai *et al.*, 1999).

However, an interesting observation arising from the study by Kawai *et al.* (1999) was that nuclear translocation of NF-κB and phosphorylation of MAPK following stimulation with LPS remained intact in macrophages from MyD88-deficient mice, albeit with delayed kinetics compared with macrophages from wild-type mice. Furthermore, another study by the same group showed that TLR4 (but not TLRs 1, 2, 5 or 6) could induce interferon-regulatory factor 3 (IRF3), a transcription factor essential for the production of IFN-β and the antiviral response (Kawai *et al.*, 2001). These findings suggest that TLR4 can also activate pathways independent of the activity of MyD88.

The MyD88-dependent signalling pathway described above is shared by all members of the TLR family, and results in the induction of a core set of responses. However, TLR4 is capable of inducing certain signalling pathways independent of this adapter, and these are described in the following section.

1.4.1.5. Myeloid-differentiation factor (MyD88)-independent TLR4 signalling

Evidence is accumulating that MyD88-independent activation of the LPS-TLR4 signalling pathway is of biological importance. For example, dendritic cells from MyD88-deficient mice showed enhanced expression of co-stimulatory molecules and increased T-cell stimulatory activity in response to LPS, suggesting

that LPS-induced DC maturation depends on a MyD88-independent pathway (Kaisho *et al.*, 2001).

A second adapter molecule known as TIR domain-containing adapter protein (TIRAP) or MyD88-adapter-like (Mal) was identified by two independent groups, and a dominant-negative form of this adapter was shown to selectively inhibit TLR4 signalling (Fitzgerald *et al.*, 2001; Horng *et al.*, 2001). TIRAP-deficient mice were found to be impaired in NF-κB and MAP kinase activation, and in cytokine production, but their expression of DC maturation markers and induction of IRF3 were intact in response to LPS (Horng *et al.*, 2002; Yamamoto *et al.*, 2002a), suggesting the involvement of yet another adapter protein. The precise role of TIRAP/Mal in TLR4 signalling remains to be determined.

A third TIR-containing adapter molecule, known as TIR-domain containing adapter inducing IFN-β (TRIF) or TIR-containing adapter molecule (TICAM)-1, has recently been identified (Yamamoto *et al.*, 2002b; Oshiumi *et al.*, 2003). The dominant-negative TRIF/TICAM-1 was shown to inhibit activation of NF-κB by TLR2, TLR3, TLR4 and TLR7, and also IFN-β promoter activation by TLR3 (Yamamoto *et al.*, 2002b). Indeed, a study carried out using TRIF/TICAM-1 knockout mice has indicated that this adapter is physiologically essential for TLR3-mediated signalling pathways (Yamamoto *et al.*, 2003). With regard to TLR4-medated signalling, TRIF/TICAM-1 has been shown to be involved in the LPS-induced MyD88-independent pathway, since cells from TRIF/TICAM-1-deficient mice are defective in TLR-4 mediated IFN-β expression and IRF3 activation in response to LPS (Yamamoto *et al.*, 2003). It has been proposed that the MyD88-dependent pathway leads to early activation of NF-κB and MAPK, while the MyD88-independent pathway causes delayed activation of NF-κB and MAPK (Fujihara *et al.*, 2003). In support of this concept, Yamamoto *et al.* (2003) demonstrated that TRIF/TICAM-1-deficient macrophages show normal activation of NF-κB and MAPK in response to LPS, whereas cells deficient in both TRIF/TICAM-1 and MyD88 showed complete loss of activation of NF-κB and MAPK (Yamamoto *et al.*, 2003).

At the time of writing, research surrounding these novel TLR4-associated adapter proteins is very much in its infancy, and future work will help elucidate the pathways downstream of the TLR4-LPS receptor complex.

1.4.2. TLR4-INDEPENDENT MECHANISMS OF LPS RECOGNITION

Although TLR4 has now been established as an essential component in the recognition of LPS, several reports have indicated that LPS can also be recognised independently of TLR4. These mechanisms are discussed briefly in the following sections.

1.4.2.1. Integrin CD11/CD18

The CD11/CD18 β2 integrins are a family of heterodimeric glycoproteins expressed on leukocytes such as monocytes, macrophages and neutrophils. The CD18 β-subunit associates noncovalently with 1 of 3 subunits: CD11a (LFA-1), CD11b (membrane attack complex (MAC)-1 or complement receptor (CR) 3) or CD11c (CR4) (Hynes, 1987). A study by Ingalls and Golenbock (1995) demonstrated that LPS non-responsive CHO cells acquired serum-independent responses to Gram-negative bacteria and LPS when transfected with CD11c/CD18, suggesting that this integral membrane protein may function as a receptor for LPS. In comparison to CHO cells transfected with CD14, responses in CHO/CD11c cells were slower and required higher LPS concentrations for maximal response (Ingalls and Golenbock, 1995). More recently, Perera *et al.* (2001) have suggested that interaction of CD11b/CD18 with TLR4 and CD14 in murine macrophages is required in order to induce the full panel of LPS-inducible genes; this study proposed that, in the absence of either CD11b/CD18 or CD14, the engagement of TLR4 by LPS delivers some, but not all (e.g. absence of cyclooxygenase 2), of the signals necessary for optimal gene expression (Perera *et al.*, 2001). This model postulates that individual receptors (TLR4, CD14 and CD11b/CD18) converge to form a multimeric receptor complex capable of inducing intricate patterns of LPS-mediated signalling.

1.4.2.2. LPS-activation cluster

A recent study carried out by Triantafilou *et al.* (2001) identified a structurally heterogeneous complex of four receptors that can bind LPS. This complex is composed of heat shock proteins (HSP) 70 and 90, chemokine receptor 4 (CXCR4) and growth differentiation factor 5 (GDF5) (Triantafilou *et al.*, 2001). Incubation with antibodies against any of the four identified receptors before LPS stimulation abrogated LPS-induced TNF-α secretion, likely due to sterical interference with other components of the receptor complex (Triantafilou *et al.*, 2001). This group of investigators have proposed the following model: LBP transfers LPS to mCD14, and the intercalated LPS is released into lipid membrane; the LPS then binds to the four-receptor complex as above, and signal-transducing molecules such as TLR4-MD-2 and CD11/CD18 are recruited into the activation cluster, triggering multiple signalling cascades (Triantafilou and Triantafilou, 2002). This new model of LPS recognition suggests that the mechanisms of action of a couple of receptor molecules may be an over-simplified model of LPS-induced activation, and may provide some interesting new insights in the future.

1.4.2.3. Cytosolic receptors – NOD proteins

In addition to TLRs, another family of cytoplasmic proteins known as NOD (nucleotide-binding oligomerisation domain) proteins have recently been implicated as cytosolic receptors for specific bacterial components, including LPS (reviewed by Inohara and Nunez, 2003).

NODs show structural homology to a class of proteins (R proteins) that are encoded by plant disease-resistance genes. The two mammalian NOD proteins, NOD1 (also known as caspase-recruitment domain (CARD) 4) and NOD2 (also known as CARD15), are highly homologous. They are composed of a variable amino-terminal effector-binding domain (EBD) containing CARD, a centrally located NOD that mediates self-oligomerisation (shown to be required for activation of downstream effector molecules (Inohara and Nunez, 2001)), and a carboxy-

terminal ligand-recognition domain (LRD) containing leucine-rich repeats, a motif also present in TLRs (Inohara and Nunez, 2003). The major structural difference between NOD1 and NOD2 is the presence of two amino-terminal CARDs in NOD2, compared with one in NOD1 (Ogura *et al.*, 2001b). The expression of NOD1 is ubiquitous in adult tissues (Bertin *et al.*, 1999; Inohara *et al.*, 1999), while NOD2 expression has been demonstrated in myelomonocytic, dendritic and epithelial cells (Ogura *et al.*, 2001b; Gutierrez *et al.*, 2002). Interestingly, mutations in the gene encoding NOD2 have been identified as the defects at the *IBD1* locus that are associated with the chronic inflammatory bowel disease Crohn's disease (Ogura *et al.*, 2001a).

Both NOD1 and NOD2 utilise a common downstream molecule known as RICK (also called RIP2 or CARDIAK), a CARD-containing protein kinase; both NODs physically associate with RICK through homophilic CARD-CARD interactions (Bertin *et al.*, 1999; Inohara *et al.*, 1999; Ogura *et al.*, 2001b). An intermediate region that is located between the CARD and the kinase domain of RICK has been shown to interact with IKKγ, the regulatory subunit of the IKK complex, thereby linking NOD1 and NOD2 to the IKK complex (Inohara *et al.*, 2000).

Expression of NOD1 or NOD2 was shown to confer LPS responsiveness (NF-κB activation) in HEK cell lines which do not normally respond to LPS, without the need for TLR4, MyD88 and TRAF-6 (Inohara *et al.*, 2001; Ogura *et al.*, 2001a). Moreover, NOD1 mediated the activation of NF-κB and c-Jun N-terminal kinase (JNK) by the invasive Gram-negative organism *Shigella flexneri* (Girardin *et al.*, 2001). NOD1 and NOD2 have also been shown to recognise LPS and/or peptidoglycan through their carboxy-terminal LRRs (Inohara *et al.*, 2001; Ogura *et al.*, 2001a; Bonen *et al.*, 2003). It has been suggested, however, that findings related to LPS recognition may be unreliable due to contamination of commercial LPS preparations with other bacterial cell wall components (Royet and Reichhart, 2003). With this in mind, several studies have recently demonstrated that NOD2 recognises muramyl dipeptide derived from peptidoglycan of Gram-negative and Gram-positive

bacteria (Girardin *et al.*, 2003b; Inohara *et al.*, 2003), and that NOD1 detects a muropeptide present in Gram-negative but not Gram-positive peptidoglycan (Chamaillard *et al.*, 2003; Girardin *et al.*, 2003a). Additionally, Chamaillard *et al.* (2003) demonstrated that HEK cells transfected with TLR4, but not NOD1 or NOD2, responded to purified intact *E. coli* lipid A with activation of NF-κB. These results indicate that NOD1, NOD2 and TLR4 may recognise different bacterial PAMPs, and at the time of writing a role for the mammalian NODs in LPS recognition is the subject of much debate. However, it is possible that intracellular detection of LPS by NOD proteins could represent a novel pathogen recognition pathway distinct from TLR4-mediated LPS recognition.

1.5. ANTIMICROBIAL PEPTIDES – EFFECTOR MOLECULES OF INNATE IMMUNITY

1.5.1. Overview

Antimicrobial peptides (AMPs) are now recognised as one of the most important elements of the innate immune system. The term 'antimicrobial peptides' describes a diverse and evolutionarily ancient group of molecules which are ubiquitous among all eukaryotes, including mammals, amphibians, insects, plants and protozoa (Hancock and Diamond, 2000). AMPs are ribosomally-synthesised, gene-encoded peptides, meaning that one gene encodes one peptide (Koczulla and Bals, 2003). Although the existence of these molecules has been known for decades, interest in their roles in immunity has recently accelerated rapidly. Moreover, the increase in bacterial resistance to conventional antibiotics has accentuated the requirement for a novel class of drugs to treat infections, and AMPs have been considered as interesting candidates for drug discovery. AMPs represent attractive candidates since they are active against antibiotic-resistant bacteria, and rarely induce bacterial resistance themselves (Hancock and Scott, 2000). However, while these peptides do not rapidly induce resistance *in vitro*, a variety of resistance mechanisms have been reported in clinical isolates; these mechanisms will not be discussed here, but have been the subject of recent reviews by Peschel (2002) and Yeaman and Yount (2003).

The definition of an AMP has been loosely applied to any peptide with the capacity to inhibit microbial growth (Gallo and Nizet, 2003), although more specifically to any polypeptide of fewer than 100 amino acids (aa) that displays antimicrobial activity at physiological concentrations, under conditions prevailing in the tissues of origin (Ganz, 2003). Although larger antimicrobial molecules such as lysozyme, lactoferrin, bacteridal/permeability-increasing protein (BPI), and the four-disulphide core proteins elafin and SLPI also play important roles in innate immune defence, they cannot by definition be regarded as antimicrobial 'peptides' and as such are not discussed in this section. However, BPI is discussed in more depth in Chapter 3, while elafin and SLPI are described in section 1.6. of this chapter.

AMPs may exhibit potent killing or inhibition of a broad spectrum of microorganimsms, including Gram-negative and Gram-positive bacteria, fungi and certain viruses. In this regard, AMPs have emerged as key factors for inhibition of microbial proliferation prior to recruitment of cellular immune defence mechanisms such as macrophages and neutrophils (Gallo and Nizet, 2003). Most of these peptides are highly cationic, a property which is important for the initial interaction between the peptide and the anionic microbial membrane preceding membrane disruption and cell lysis, and have a structure that organises charged residues separately from hydrophobic residues. Four main mechanisms of peptide-mediated membrane permeabilisation have been described: the barrel-stave model, worm-hole model, the carpet model, and selective ion channel formation (Ganz and Lehrer, 1999). Moreover, the selectivity of AMPs for prokaryotic cells seems to depend on fundamental differences in the lipid composition of microbial and host cell membranes (Yeaman and Yount, 2003).

AMPs are now known to contribute to innate immunity via activities distinct from antimicrobial activity, such as binding to and modulating the activity of LPS (Gough *et al.*, 1996); as mentioned in section 1.3.2.2., cationic peptides can completely displace the LPS-associated divalent cations which stabilise the structure of the bacterial outer membrane (Hancock and Chapple, 1999). Additionally, these

peptides may act as multifunctional mediators of immunity, inflammation and wound repair.

In humans and other mammals, the two main AMP families are defensins and cathelicidins; these two families are discussed in the following sections.

1.5.2. Defensins

Defensins are a family of evolutionarily related vertebrate peptides (typically 30-45 residues) with a characteristic β-sheet-rich fold and a framework of six disulphide-linked cysteine residues (Lehrer and Ganz, 2002; Ganz, 2003). The two main defensin subfamilies, α- and β-defensins, can be distinguished based on the localisation and pairing of the six cysteines in the disulphide bridges. A third defensin subfamily, characterised by a cyclic structure, has recently been identified in rhesus macaque monkey leukocytes and named the θ-defensins (Tang *et al.*, 1999). However, this subfamily is inactive in humans due to gene mutations that encode premature stop codons (Cole *et al.*, 2002).

1.5.2.1. α-defensins

α-defensins contain three disulphide bridges in a Cys1-Cys6, Cys2-Cys4, Cys3-Cys5 alignment (Koczulla and Bals, 2003). Six α-defensins have been identified in humans to date. Human neutrophil peptides 1-4 (HNP 1-4) are localised in azurophilic granules of neutrophils, in which they are the principal protein and contribute to the oxygen-independent killing of phagocytosed microorganisms (Ganz *et al.*, 1985). Moreover, they may be secreted by neutrophils and thus accumulate to high concentrations in the extracellular milieu during infection and inflammation (Aarbiou *et al.*, 2002b). The two other α-defensins, human defensins 5 and 6 (HD-5 and -6), are primarily expressed in Paneth's cells of the small intestinal crypts (Jones and Bevins, 1992). An important role for Paneth cell defensins in host defence against intestinal infection has been demonstrated by two key studies: mice deficient in matrilysin (matrix metalloproteinase (MMP) 7), a tissue metalloproteinase

involved in the processing of mouse Paneth cell defensins (cryptdins), displayed increased sensitivity to intestinal infections (Wilson *et al.*, 1999), while HD-5 transgenic mice showed enhanced resistance against enteric salmonellosis (Salzman *et al.* 2003).

α-defensins are generally encoded as a 90-100 amino acid prepropeptide, containing an N-terminal signal sequence (~19aa), an anionic propiece (~45aa), and a C-terminal mature cationic defensin (~30aa) (Ganz, 2003). After signal peptide cleavage, the anionic propiece neutralises the cationic mature peptide, which may be important for folding and prevention of interaction with the host membrane (Valore *et al.*, 1996).

1.5.2.2. β-defensins

β-defensins contain three disulphide bridges in a Cys1-Cys5, Cys2-Cys4, Cys3-Cys6 alignment, and are generally slightly larger than α-defensins (36-42aa in length) (Koczulla and Bals, 2003). Four human β-defensins have been characterised to date, namely human β-defensins (HBD)-1 to –4. Based on recent genomic studies which revealed additional defensin gene clusters, it is likely that several more HBDs exist and play a role in innate defence (Schutte *et al.*, 2002; Kao *et al.*, 2003). In comparison to α-defensins, the structure of β-defensin precursors is simpler, consisting of a signal sequence, a short or absent propiece, and the C-terminal mature defensin peptide (Ganz, 2003). The processing of β-defensins is likely similar to that of α-defensins, but has not been clarified to date.

The majority of studies on β-defensin expression have centred on their expression in epithelial tissues such as the mucosa of the lung, gastrointestinal tract and the skin (Schutte and McCray, 2002). For example, HBD-1 and HBD-2 have been detected in airway secretions in concentrations reaching the μg/ml range (Bals *et al.*, 1998b; Singh *et al.*, 1998). However, HBDs may also be expressed by other cell types such as monocytes/macrophages and dendritic cells (Duits *et al.*, 2002).

Interestingly, epithelial expression of HBD-1 appears to be constitutive, while the expression of HBDs 2-4 has been shown to be inducible by bacteria, viruses, neutrophil elastase and inflammatory cytokines, providing further evidence for their important role in the initial stages of innate immunity (Garcia *et* al., 2001a, 2001b; Harder *et al.*, 2000; Harder *et al.*, 2001; Duits *et al.*, 2003a; Griffin *et al.*, 2003). Recent studies have also suggested that, while epithelial cells may display a low responsiveness to microbial products, this response may be augmented by macrophages to boost the local antimicrobial shield; TNF-α and IL-1β secreted by LPS-stimulated macrophages markedly enhanced the expression of HBD-2 by skin and pulmonary epithelial cells (Liu *et al.*, 2003; Tsutsumi-Ishii *et al.*, 2003).

A range of murine β-defensins have also been identified and characterised (Morrison *et al.*, 2003); these will be discussed in more detail in Chapter 6 of this thesis.

1.5.3. Cathelicidins

Cationic peptides of the cathelicidin family are approximately 12-100 aa in length and, similar to the defensins, are produced as an inactive precursor; these pre-pro-peptides consist of an N-terminal signal peptide, a highly conserved prosequence and a structurally variable C-terminal mature antimicrobial peptide (Zanetti *et al.*, 1995). The prosequence (99-114aa) is known as the 'cathelin' domain due to its homology with cathelin, a porcine neutrophil protein that inhibits the protease cathepsin L (Bals and Wilson, 2003; Nizet and Gallo, 2003). The cathelin pro-peptide must be removed from the mature peptide in order to unleash the antimicrobial activity, and in the case of the human peptide LL-37 this is achieved by extracellular cleavage of its precursor hCAP-18 by proteinase 3 in neutrophils (Sorensen *et al.*, 2001) or by gastricsin in seminal plasma (Sorensen *et al.*, 2003). Interestingly, however, a recent study has demonstrated that the cathelin pro-peptide of hCAP-18/LL-37 also possesses antimicrobial activity, in addition to cathepsin L-inhibitory activity (Zaiou *et al.*, 2003).

Whereas a range of cathelicidins have been identified in species such as cattle and pigs, hCAP-18/LL-37 represents the only known human cathelicidin, while a homologue of LL-37 known as CRAMP (cathelin-related antimicrobial peptide) is the sole murine cathelicidin (Gallo *et al.*, 1997; Nizet and Gallo, 2003). The important antimicrobial activities of these molecules has been demonstrated by several studies. Firstly, adenovirus-mediated overexpression of hCAP-18/LL-37 in a cystic fibrosis xenograft model restored the disease-specific defect in antimicrobial activity to normal levels (Bals *et al.*, 1999b). Secondly, murine models of pneumonia and endotoxic shock further demonstrated that adenovirus-mediated hCAP-18/LL-37 overexpression could reduce pulmonary bacterial load, inflammation and endotoxaemia-induced mortality (Bals *et al.*, 1999c). Finally, mice deficient in CRAMP were found to have an increased susceptibility to skin infections by Group A *Streptococci* (Nizet *et al.*, 2001).

In humans, hCAP-18/LL-37 appears to be produced predominantly by neutrophils, but is also expressed in various squamous epithelia, respiratory epithelia, keratinocytes in inflamed skin, specific lymphocyte and monocyte populations, and mast cells (Frohm *et al.*, 1997; Sorensen *et al.*, 1997; Bals *et al.*, 1998c; Frohm *et al.*, 1999; Agerberth *et al.*, 2000; Di Nardo *et al.*, 2003). Expression of hCAP-18/LL-37 can be upregulated in several cell types by inflammatory mediators, such as IL-6 (Frohm *et al.*, 1997). Conversely, studies have suggested that *Shigella* species may protect themselves from enteric innate immunity by downregulating expression of both HBD-1 and hCAP-18/LL-37 (Islam *et al.*, 2001).

1.5.4. Activities of defensins/cathelicidins distinct from antimicrobial action

In addition to antimicrobial activity, cationic antimicrobial peptides have also been found to play a number of further roles in the immune response.

As mentioned previously, antimicrobial peptides (including defensins and cathelicidins) can bind directly to LPS (Larrick *et al.*, 1995; Gough *et al.*, 1996; Turner *et al.*, 1998; Scott *et al.*, 1999b; Scott *et al.*, 2000b) and are capable of blocking the ability of LPS to stimulate the production of TNF-α, IL-6 and other

inflammatory mediators *in vitro* (Gough *et al.*, 1996; Scott *et al.*, 2000b; Nagaoka *et al.*, 2001). Studies have also suggested that these effects may be mediated, at least in part, by the ability to inhibit binding of LPS to LBP, or of LPS to CD14-positive cells (Scott *et al.*, 2000b; Nagaoka *et al.*, 2001). Furthermore, LL-37 and LL-37 analogues can neutralise the effects of LPS *in vivo*, decreasing TNF-α production and protecting mice against the lethal effects of endotoxaemia (Kirikae *et al.*, 1998; Bals *et al.*, 1999c; Nagaoka *et al.*, 2001; Scott *et al.*, 2002).

Antimicrobial peptides have also been shown to activate epithelial cells. Neutrophil defensins (HNP 1-3) induce release of the neutrophil-attractant chemokine IL-8 from human alveolar A549 and bronchial epithelial cells (van Wetering *et al.*, 1997b), and also of the low molecular weight proteinase inhibitor/four-disulphide core protein SLPI from bronchial epithelial cells (van Wetering *et al.*, 2000a). Similarly, LL-37 has been found to increase IL-8 production by A549 cells, and also to augment expression of the chemokine monocyte chemoattractant protein (MCP)-1 and chemokine receptors CXCR-4, CCR2 and IL-8RB in macrophages (Scott *et al.*, 2002). The latter study also demonstrated that LL-37 can effectively downregulate the LPS-mediated release of TNF-α in macrophages, suggesting that this cathelicidin could prevent overwhelming macrophage-mediated inflammation, while promoting local leukocyte responses to infection (Scott *et al.*, 2002).

HNPs have been implicated in repair of injured airway epithelium, since HNP 1-3 can induce lung epithelial cell proliferation *in vitro* via an epidermal growth factor receptor (EGFR)-independent MAP kinase signalling pathway (Aarbiou *et al.*, 2002a). HNPs are also mitogenic for retinal epithelial cells and fibroblasts (Murphy *et al.*, 1993). However, high (μM) concentrations of neutrophil defensins have been shown to be cytotoxic to epithelial cells, and decrease proliferation (Okrent *et al.*, 1990; van Wetering *et al.*, 1997a; Aarbiou *et al.*, 2002a). It has also been demonstrated that LL-37 can activate epithelial cells by activation of the MAP kinase/extracellular signal-regulated kinase (ERK1/2) pathway, although LL-37's effects are mediated via metalloproteinase-dependent transactivation of EGFR

(Tjabringa *et al.*, 2003). Additionally, LL-37 may play a role in angiogenesis and arteriogenesis by inducing proliferation and formation of vessel-like structures in endothelial cells, through interaction with the G protein-coupled, seven transmembrane receptor formyl peptide receptor-like 1 (FPRL1) (Koczulla *et al.*, 2003).

Further modulation of the innate immune response by defensins and cathelicidins is evidenced by the observations that HNPs can upregulate TNF-α and IL-1β production, and downregulate production of the anti-inflammatory cytokine IL-10, by *S. aureus*-activated monocytes (Chaly *et al.*, 2000), while HNPs, HBD2 and LL-37 induce the activation and degranulation of mast cells, resulting in release of histamine and prostaglandin D_2 (PGD_2) (Befus *et al.*, 1999; Niyonsaba *et al.*, 2001). These effects may serve to amplify local inflammatory defences against microbial invasion.

Interestingly, findings regarding roles for defensins and cathelicidins in cellular chemotaxis have suggested that they may also play a role in linking innate immunity with the adaptive immune response to infection. Human neutrophil defensins have been shown to be chemotactic for human monocytes, T cells and immature dendritic cells (Territo *et al.*, 1989; Chertov *et al.*, 1996; Yang et *al.*, 2000a), while the β-defensins appear to act as chemoattractants for immature dendritic cells, memory T cells and macrophages (Yang *et* al., 1999; Garcia *et al.*, 2001a, 2001b). The receptor used by β-defensins to mediate their chemotactic effects has been identified as the CC chemokine receptor 6 (CCR6) (Yang *et al.*, 1999), whereas a specific receptor for the HNPs has not yet been identified. Also of interest in this regard is the recent observation that murine β-defensin 2 may act directly on immature dendritic cells as an endogenous ligand for TLR4, inducing up-regulation of co-stimulatory molecules and dendritic cell maturation (Biragyn *et al.*, 2002). LL-37 exhibits chemotactic activity towards human monocytes, T cells and neutrophils through binding to the aforementioned FPRL1 (Yang *et al.*, 2000b), and also chemoattracts mast cells (Niyonsaba *et al.*, 2002). Another cathelicidin, porcine PR-39, has been shown to be chemotactic for neutrophils (Huang *et al.*, 1997).

The use of chemotactic receptors by defensins and cathelicidins suggests that they may provide a functional overlap with chemokines, and it is interesting to note that recent findings have described defensin-like antimicrobial activities of chemokines (Durr and Peschel, 2002).

It can therefore be concluded that AMPs play a variety of roles in the innate immune response besides their antimicrobial function, and these activities may encompass modulation of inflammatory processes, cell proliferation, wound repair and immune cell chemotaxis.

1.6. LOW MOLECULAR WEIGHT PROTEINASE INHIBITORS/ FOUR-DISULPHIDE CORE PROTEINS

1.6.1. Overview

During inflammatory responses instigated by bacterial products such as LPS, excessive or unregulated release of proteolytic enzymes (normally utilised in defence against microbial pathogens) by inflammatory and stromal cells can lead to severe tissue damage and dysfunction (Hubbard *et al.*, 1991). The major source of serine proteases is the neutrophil, and human neutrophil elastase (HNE) is a significant instigator of tissue degradation (Weiss, 1989). Its potential substrates include almost all components of the extracellular matrix, such as elastin, collagen (types I-IV) and proteoglycan (Kawabata *et al.*, 2002). Other serine proteases released from the azurophilic granules of neutrophils during inflammatory processes include proteinase-3 and cathepsin G (Hubbard *et al.*, 1991). Accordingly, inhibitors of proteinase activity comprise an important part of the innate immune system. For example, three distinct anti-elastases are known to comprise the 'anti-elastase shield' in the lung: α1-proteinase inhibitor (α1-PI), SLPI and elafin. While α1-PI is synthesised principally in the liver by hepatocytes and released into the circulation, SLPI and elafin are produced locally at mucosal sites (Sallenave *et al.*, 1999b). SLPI and elafin together form the antileukoprotease (ALP) family, and are often referred to as 'low molecular weight proteinase inhibitors' or 'four-disulphide core proteins' (Sallenave, 2000). SLPI and elafin are structurally similar, and in fact share

approximately 40% sequence homology (Wiedow *et al.*, 1990; Sallenave and Ryle, 1991; Sallenave and Silva, 1993). These molecules are now known to possess activities distinct from their roles as inhibitors of proteolytic enzymes. The following sections will discuss these two molecules in more depth, with particular emphasis on the current knowledge surrounding elafin, since the work described in this thesis was primarily concerned with investigating novel roles for elafin in the innate immune response.

1.6.2. Secretory leukocyte proteinase inhibitor (SLPI)

SLPI (which has also been known as antileukoprotease, mucus proteinase inhibitor and bronchial mucus inhibitor) is a 107 amino acid, single-chain, non-glyosylated cationic protein, with a molecular weight of 11.7kDa (Seemuller *et al.*, 1986; Thompson and Ohlsson, 1986). The SLPI molecule is arranged in a compact, 'boomerang' shape, and characteristically contains two structural domains each containing four disulphide bridges (Grutter *et al.*, 1988). The NH_2-terminal domain is composed of residues 1-54, while the COOH-terminal domain consists of residues 55-107 (Seemuller *et al.*, 1986). The antiproteinase active site resides in the COOH-terminal domain; the active residue is leucine at position 72, while the methionine residue at position 73 has been shown to be susceptible to oxidation, which renders SLPI inactive towards HNE (Rudolphus *et al.*, 1991). SLPI is capable of inhibiting HNE, cathepsin G, trypsin, chymotrypsin and mast cell chymase, but does not inhibit proteinase 3 (reviewed by Sallenave *et al.*, 1999b). The size and compact structure of SLPI may contribute to its ability to access the gap between neutrophil and substrate (Rice and Weiss, 1990), and human SLPI has been detected in association with elastic fibres in pulmonary connective tissue (Willems *et al.*, 1986).

SLPI has been shown to be widely distributed in human tissues, and is produced at mucosal sites in respiratory, nasal, salivary, cervical and semenal secretions (reviewed by Sallenave *et al.*, 1999b), as well as by inflammatory cells such as macrophages and neutrophils (Jin *et al.*, 1997; Sallenave *et al.*, 1997; Mihaila and Tremblay, 2001). Along with elafin, SLPI may be regarded as an inducible 'alarm' defence molecule, since its production is enhanced by early inflammatory

signals such as LPS, TNF-α, IL-1β, HNE, cathepsin G and also by neutrophil defensins (Sallenave *et al.*, 1994; Jin *et* al., 1998; van Wetering *et al.*, 2000a; van Wetering *et al.*, 2000b). Conversely, anti-inflammatory cytokines such as transforming growth factor (TGF)-β can down-regulate the production of SLPI (Jaumannn *et al.*, 2000).

While protection of tissues from protease-mediated damage at inflammatory sites is considered to be a major physiological role of SLPI, recent studies have shown that this molecule functions as more than just a protease inhibitor.

It has been demonstrated that SLPI possesses broad spectrum antimicrobial activity against a range of pathogens, including Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*), Gram-positive bacteria (*Staphylococcus aureus*, *S. epidermidis*), fungi (*Aspergillus fumigatus*, *Candida albicans*), and retroviruses (HIV-1) (McNeely *et al.*, 1995; Hiemstra *et al.*, 1996; Tomee *et al.*, 1997; Wiedow *et al.*, 1998). Hiemstra *et al.* (1996) have further indicated that the anti-bacterial activity of SLPI resides in its NH_2-terminal domain.

SLPI may also play a role in normal wound healing, since skin wounds in SLPI-deficient mice healed slowly with impaired matrix accumulation, and persistence of inflammatory cells (Ashcroft *et al.*, 2000).

Furthermore, anti-inflammatory functions for SLPI have been described by a number of studies. Firstly, Mulligan *et al.* (1993) used *in vivo* rat models to demonstrate that exogenous SLPI could suppress IgG immune complex-induced alveolitis, which is characterised by neutrophil accumulation and lung vascular leak. Using the same model of acute lung injury, it has since been shown that the anti-inflammatory effects of SLPI may be due to inhibition of intrapulmonary activation of NF-κB, coupled with augmentation of the levels of IκBβ (Lentsch *et al.*, 1999a). Mulligan *et al.* (2000) have further suggested that these effects are related to the trypsin-inhibiting activity of SLPI, and may be directed at the intracellular proteases involved in degradation of IκB proteins by the 26S proteasome/MPC (Mulligan *et*

al., 2000). Additionally, similar immune complex models have suggested that endogenous rat SLPI may play a role in preventing lung injury by inhibiting generation of the neutrophil-chemotactic inflammatory mediator, C5a (Gipson *et al.*, 1999). In a murine model of hepatic ischemia/reperfusion injury, SLPI also reduced lung and liver damage and diminished neutrophil accumulation, accompanied by decreased serum levels of TNF-α and the CXC chemokine macrophage inflammatory protein (MIP)-2 (Lentsch *et al.*, 1999b); moreover, SLPI suppressed NF-κB activation in liver in this model (Lentsch *et al.*, 1999b).

Of particular relevance to the work in this thesis are observations concerning the activity of SLPI in modulating LPS-mediated inflammatory responses. Interestingly, SLPI has been shown to bind directly to LPS, prevent binding of LPS to sCD14, and slow the uptake of LPS-sCD14 complexes by macrophages (Ding *et al.*, 1999).

SLPI was also shown to inhibit the signalling pathways leading to generation of matrix metalloproteinases in LPS-stimulated monocytes (Zhang *et al.*, 1997). The work of Ding and colleagues has subsequently been instrumental in delineating a role for SLPI in dampening the LPS-mediated activation of macrophages. Having demonstrated that SLPI is inducible by LPS and is constitutively overexpressed in LPS-hyporesponsive macrophages, their work also indicated that transfection of macrophages with SLPI could suppress LPS-induced activation of NF-κB and production of nitric oxide and TNF-α (Jin *et al.*, 1997; Jin *et al.*, 1998). In an extension of the latter, Zhu *et al.* (1999) demonstrated that transfection of macrophages with a non-secretory from of SLPI effectively suppressed macrophage nitrite and TNF-α secretion in response to LPS, while exogenous SLPI had no effect; these data have suggested that SLPI's anti-inflammatory effects are mediated intracellularly (Zhu *et al.*, 1999). These observations have been supported by Sano and colleagues, who showed that, while exogenous SLPI could augment production of the anti-inflammatory cytokines IL-10 and TGF-β by LPS-stimulated macrophages, secretion of TNF-α was not inhibited (Sano *et al.*, 2000; Sano *et al.*, 2003). In this regard, Taggart *et al.* (2002) recently demonstrated that SLPI can

prevent NF-κB activation in LPS-stimulated monocytes by precluding degradation of IRAK, IκBα and IκBβ, and have suggested that this effect is educed via inhibition of the ubiquitin-proteasome pathway (Taggart *et al.*, 2002).

Finally, a role for SLPI in attenuating excessive LPS-mediated inflammatory responses has been suggested by a recent study carried out using SLPI-deficient mice, which showed a higher mortality from endotoxic shock than did wild-type mice (Nakamura *et al.*, 2003). This study made the additional observation that $SLPI^{-/-}$ macrophages released higher levels of IL-6 in response to LPS, and demonstrated increased activation of NF-κB, when compared with $SLPI^{+/+}$ macrophages (Nakamura *et al.*, 2003). It is also interesting to note that serum levels of SLPI are elevated in human sepsis and experimental endotoxaemia, suggesting a role in regulation of LPS-mediated inflammation in humans *in vivo* (Grobmyer *et al.*, 2000). SLPI levels are additionally increased in the bronchoalveolar lavage (BAL) fluid of patients with sepsis-associated acute respiratory distress syndrome (ARDS), an acute lung inflammatory condition characterised by a predominantly neutrophilic alveolitis (Sallenave *et al.*, 1999a).

The LPS-modulating activities of SLPI will be discussed in more detail within the 'Results' sections to follow.

1.6.3. Elafin

Elafin (which has also been known as elastase-specific inhibitor (ESI), skin-derived antileukoprotease (SKALP) or trappin-2) was first identified by Hochstrasser *et al.* (1981) and Kramps and Klasen (1985) as a low molecular weight anti-elastase present in bronchial secretions. The elastase-specific inhibitor was subsequently isolated and purified from the sputum of patients with chronic bronchitis, and characterised as a fast-acting inhibitor of HNE and porcine pancreatic elastase (PPE) (Sallenave and Ryle, 1991). ESI/elafin shared considerable homology with SLPI, although the scissile bond forming the active inhibitory site of ESI was shown to be Ala-Met (compared with Leu-Met in SLPI), and this difference is thought to be

important for the disparity in these molecules' proteinase-inhibitory spectra (Sallenave and Ryle, 1991). At around the same time, two groups independently described an elastase inhibitor purified from psoriatic skin scales and human keratinocytes, which they named elafin and SKALP in parallel but was later shown to represent the same molecule (Wiedow *et al.*, 1990; Schalkwijk *et al.*, 1990; Schalkwijk *et al.*, 1991).

The moiety isolated from sputum by Sallenave and Ryle (1991) was found to cross-react immunologically with the species described by Kramps and Klasen (1985), and to have almost complete sequence homology with the molecules purified from skin (Sallenave *et al.*, 1992). The gene encoding elafin was consequently cloned and sequenced, and shown to code for a 117 amino acid protein, of which the first 22 amino acids represent a hydrophobic signal peptide (Sallenave and Silva, 1993). The sequence of the gene for elafin, which is located on chromosome 20, showed that it is approximately 2.3kb long, is composed of three exons and two introns, and contains 5' regulatory sequences such as AP-1 and NF-κB sites (Sallenave and Silva, 1993; Molhuizen *et al.*, 1994; Schalkwijk *et al.*, 1999).

Elafin has been shown to be a more specific inhibitor of elastolytic proteinases than SLPI, since it inhibits HNE, PPE and proteinase 3 (Wiedow *et al.*, 1990; Sallenave and Ryle, 1991; Wiedow *et al.*, 1991; Ying and Simon, 2001), but does not inhibit cathepsin G, trypsin or chymotrypsin (Wiedow *et al.*, 1990; Tsunemi *et al.*, 1992). It is produced in a variety of epithelial and mucosal sites throughout the human body, such as the skin, the oral cavity, the small intestine, and the lung (Molhuizen *et al.*, 1993; Sallenave *et al.*, 1993; Nara *et al.*, 1994; Pfundt *et al.*, 1996). Lung expression of elafin may be associated with alveolar epithelium, Clara (bronchial) cells, or tracheal epithelium (Sallenave *et al.*, 1993; Nara *et al.*, 1994; Suzuki *et al.*, 2000), and elafin has been found to constitute approximately 20% of the total anti-elastase antigen retrieved from bronchoalveolar lavage fluid during health (Tremblay *et al.*, 1996). Moreover, elafin production has also been described in inflammatory cells such as neutrophils and macrophages (Sallenave *et al.*, 1997; Mihaila and Tremblay, 2001; Simpson *et al.*, 2001a). Similar to SLPI, early

inflammatory 'alarm signals', such as LPS, TNF-α, IL-1β and HNE, can switch on production of elafin (Sallenave *et al.*, 1994; Reid *et al.*, 1999; Tanaka *et al.*, 2000; van Wetering *et al.*, 2000b; Simpson *et al.*, 2001a), suggesting a role in the initial stages of the innate immune response to infection.

The elafin molecule is a 9.9kDa non-glycosylated cationic protein which, as mentioned previously, has approximately 40% sequence homology with SLPI (Wiedow *et* al., 1990; Sallenave and Ryle, 1991; Sallenave and Silva, 1993). The elafin molecule has two distinct structural domains. The COOH-terminal domain is compact due to the presence of four disulphide bonds, and contains the Ala-Met active inhibitory site (Sallenave and Ryle, 1991; Tsunemi *et al.*, 1996). As is the case with SLPI, the methionine in the active site of elafin may become oxidised with loss of function (Rudolphus *et al.*, 1991). Elafin's inhibitory activity has previously been shown to be inactivated by PPE and sputum from patients with cystic fibrosis (Sallenave *et al.*, 1995). The COOH-terminal domain of elafin is also often termed the whey acid protein (WAP) domain, leading Schalkwijk *et al.* (1999) to classify elafin as a member of the 'trappin' family, hence the name trappin-2.

The NH_2-terminal domain of elafin is characterised by repeated VKGQ motifs (Sallenave and Silva, 1993). This sequence (also termed 'cementoin' (Nara *et al.*, 1994)) acts as a substrate domain for transglutaminase, with the resultant formation of covalent isopeptide bonds between lysine and glutamine (Schalkwijk *et al.*, 1999). Transglutaminase has been shown to instigate polymerisation of elafin and the covalent binding of elafin to laminin *in vitro* (Nara *et al.*, 1994), suggesting that tissue transglutaminase may anchor elafin to extracellular matrix *in vivo* and prolong its local activity. In this regard, elafin has been identified cross-linked to keratin 1 and loricrin in human cornified cell envelope (Steinert and Marekov, 1995), while transglutaminase type 1 has been co-localised with elafin in stratified epithelia (Schalkwijk *et al.*, 1999).

Although inhibition of elastolytic activity has historically been considered to be the primary role of elafin, recent work has highlighted further properties of this molecule which propose it as a 'defensin-like' entity.

Simpson *et al.* (1999) demonstrated that elafin has antibacterial activity against Gram-negative *P. aeruginosa* and Gram-positive *S. aureus*, and further established that, while anti-elastase activity resides exclusively in the COOH-terminal domain, the majority of elafin's antimicrobial activity resides in its NH_2-terminal domain (Simpson *et al.*, 1999). The demonstration of elafin's antimicrobial activity was corroborated by Meyer-Hoffert *et al.* (2003), who showed that elafin could inhibit the growth of *P. aeruginosa*, but not *E. coli* (Meyer-Hoffert *et al.*, 2003). In an extension of these studies, augmentation of elafin in murine lungs (using an adenovirus vector encoding human elafin cDNA (Ad-elafin)) was shown to confer significant protection against injury mediated by *P. aeruginosa in vivo*, and also reduced bacterial numbers (Simpson *et al.*, 2001b). Moreover, the same study found that elafin overexpression could protect pulmonary epithelial cells against HNE and activated human neutrophils *in vitro* (Simpson *et al.*, 2001b).

Furthermore, similar to the antimicrobial peptides described previously, elafin may exhibit chemotactic activity for neutrophils. Adenovirus-mediated over-expression of elafin enhanced neutrophil migration *in vitro*, and this effect could be reduced by incorporating an anti-elafin antibody (Simpson *et al.*, 2001a). In addition, infection of murine airways with Ad-elafin significantly augmented LPS-mediated neutrophil migration *in vivo*, and was also shown to enhance LPS-induced levels of TNF-α and MIP-2 in BAL fluid (Simpson *et al.*, 2001a). Elafin has been postulated to function in this model by providing local antimicrobial protection and enhancing neutrophil influx, while protecting host tissues from subsequent HNE-mediated damage (Simpson *et al.*, 2001a). Interestingly, work carried out using transgenic mice expressing human elafin has demonstrated that these mice exhibit lower serum-to-BAL fluid ratios of pro-inflammatory cytokines (TNF-α, MIP-2 and MCP-1) than wild-type mice, following LPS administration to the lungs; this was associated with an increase in neutrophil and macrophage influx, suggesting priming of innate

responses in the lungs (Sallenave *et al.*, 2003). Conversely, when LPS was given systemically, mice expressing elafin had lower levels of serum TNF-α than wild-type mice, while peritoneal macrophages from elafin mice were hypo-responsive to LPS (Sallenave *et al.*, 2003). In this transgenic mouse model, elafin similarly appears to function to promote local immunity, but reduce systemic inflammation. The aforementioned studies will be discussed in more depth during later chapters.

In another animal model, Vachon *et al.* (2002) contrastingly showed that recombinant elafin could reduce neutrophil influx, gelatinase activity, and levels of MIP-2 and TNF-α in lung following intranasal LPS administration. These findings would suggest that elafin may play a role as a potent anti-inflammatory mediator in the lung; the discrepancies between this study and those described above may reflect differences in elafin concentration, dose of LPS administered, route of administration, and duration of the experiments. Future studies may be required to clarify these differing observations.

Other *in vivo* models have further indicated that exogenous (human) elafin can play an anti-inflammatory role by inhibiting HNE-induced acute lung injury in hamsters (Tremblay *et al.*, 2002), protecting against vein graft degeneration in rabbits by reducing the early inflammatory response associated with serine elastase activity (O'Blenes *et al.*, 2000), inhibiting smooth muscle proliferation and inflammatory cell invasion after arterial injury in mice (Zaidi *et al.*, 2000), attenuating post-cardiac transplant coronary arteriopathy and myocardial necrosis in rabbits (Cowan *et al.*, 1996), and inhibiting intimal hyperplasia caused by serine elastase activity following balloon angioplasty in rabbits (Barolet *et al.*, 2001).

At this point it seems important to note that, while recent work by Simpson *et al.* has striven to unearth possible applications for elafin gene therapy *in vivo* with the use of adenoviral vectors, the work in this thesis has been concerned primarily with *in vitro* techniques aimed at delineating elafin's activities. Adenovirus-mediated gene transfer has, however, been used here in order to facilitate genetic augmentation of elafin in primary cells and cell lines. While the author acknowledges the potential

application of gene therapy techniques to treatment of bacterial and/or LPS-mediated inflammation, strategies such as those involving the use of adenoviral vectors will not be discussed in detail here. The reader is instead referred to recent reviews on this subject by Simpson *et al.* (2000), Factor (2001) and Thomas *et al.* (2003).

1.7. SUMMARY

This introduction has outlined the importance of Gram-negative bacterial LPS in induction of inflammatory responses, and has described the major mechanisms for its recognition by the innate immune system. Small cationic molecules such as antimicrobial peptides and the low molecular weight proteinase inhibitors elafin and SLPI may play an important role in modulation of inflammatory responses *in vivo*. Several biochemical characteristics of elafin, such as low molecular weight, cationicity, heavy disulphide bonding and tissue distribution at mucosal sites, together with its antimicrobial and neutrophil-chemotactic activities, have suggested that elafin may share further properties with antimicrobial peptides. In particular, antimicrobial peptides can bind to LPS and modify its biological effects. Taken together with the previous description of SLPI's anti-endotoxin activities, these observations led us to investigate potential roles for elafin in modulating LPS-mediated inflammatory responses, using *in vitro* models. These principles generated the hypotheses central to this work.

1.8. CENTRAL HYPOTHESES

The initial hypothesis driving this work was that ***elafin may be capable of interacting directly with bacterial lipopolysaccharide,*** and that ***this property could confer to elafin the ability to modulate the pro-inflammatory activity of LPS.*** The hypothesis that elafin may bind directly to LPS was tested using a series of polyacrylamide gel-based assays, while the effects of elafin-LPS interaction on the binding of LPS to LBP were studied by ELISA. The results of these experiments are presented in Chapter 3.

Taking into consideration the findings of Chapter 3, the hypothesis that ***interaction of elafin with LPS could modulate the pro-inflammatory activity of LPS*** was tested *in vitro*. The model chosen to test this hypothesis involved stimulation of murine macrophages or bronchial epithelial cells with LPS, in the presence or absence of synthetic elafin peptides. The effects of elafin on the LPS-mediated activation of cells were assessed by measuring cytokine or chemokine production, respectively. The data generated by these assays are described in Chapter 4.

To further investigate the LPS-modulatory activities of elafin *in vitro*, the hypothesis that ***elafin gene augmentation could modulate the inflammatory response of macrophages to LPS*** was tested. The model used to test this hypothesis involved infection of murine macrophages with an adenovirus vector encoding human elafin cDNA, followed by stimulation with LPS. The results are described in Chapter 5.

The findings of Chapters 3, 4 and 5, in conjunction with elafin's characteristics as a low molecular weight cationic protein expressed primarily at epithelial sites and up-regulated in response to early inflammatory stimuli, prompted the further hypothesis that ***elafin gene augmentation could enhance the antimicrobial activity of murine epithelial cells***. The model employed to test this centred around infection of primary murine tracheal epithelium with adenovirus encoding human elafin, followed by challenge with *Pseudomonas aeruginosa* or *Staphylococcus aureus*. Results from this model are presented in Chapter 6.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Source of chemicals and reagents

Unless otherwise stated, chemicals and reagents were obtained from Sigma (Poole, Dorset, UK).

2.1.2. Synthetic human elafin peptides

Elafin peptides were produced synthetically by Albachem (Edinburgh, UK) using standardised techniques described elsewhere (Morrison *et al.*, 1998). The established gene sequence for human elafin (Sallenave and Silva, 1993) was used to derive amino acid sequences. The peptides used in this work were available in-house from the outset, and have been described previously by Simpson *et al.* (1999). Three peptides were used for these studies, namely full-length elafin (H_2N-^{1}AVT.....^{95}Q-COOH), the NH_2-terminal domain (H_2N-^{1}AVT.....^{50}K-COOH) and the COOH-terminal domain (H_2N-^{51}PGS.....^{95}Q-COOH). The molecular weights of elafin moieties, determined by mass spectrometry (Albachem), were 9925 Da for full-length elafin, 5172 Da for the NH_2-terminal domain and 4776 Da for the COOH-terminal domain.

2.1.3. Polyclonal rabbit anti-human elafin antibody

Polyclonal rabbit anti-human elafin IgG, prepared from antiserum raised against a COOH-terminal 2.5kDa fraction of human elafin (Sallenave *et al.*, 1992), was available in-house from the outset of this work. Biotinylated rabbit anti-human elafin IgG was also available in-house.

2.1.4. Adenoviral constructs

Six E1-, partially E3-deleted adenoviral vector constructs of serotype 5 were used, and these vectors were available in-house from the outset of this work. The vectors used were: Ad-elafin, encoding the cDNA for human elafin; Ad-lacZ, encoding the lacZ reporter gene; Ad-mSLPI, encoding the cDNA for murine SLPI; Ad-dl70/3, an empty vector encoding no transgene; Ad-IκB, encoding the cDNA for an NF-κB super-repressor mutant of the cytoplasmic protein IκBα; and Ad-m-eotaxin, encoding the cDNA for the murine CC chemokine eotaxin. The techniques used to generate these adenovirus vectors have been described in detail elsewhere (Hitt *et al.*, 1995; Addison *et al.*, 1997; Jobin *et al.*, 1998; Sallenave *et al.*, 1998; Wang and Huang, 2000). With the exception of Ad-IκB, which incorporates the human cytomegalovirus (CMV) promoter (Jobin *et al.*, 1999), the transgene in each vector is located downstream of a fragment of the murine cytomegalovirus (MCMV) immediate early promoter (Dorsch-Hasler *et al.*, 1985). While the Ad-elafin, Ad-lacZ, Ad-dl70/3 and Ad-m-eotaxin vectors encode a single transgene driven by a 1.4kb fraction of the MCMV promoter, Ad-mSLPI is a bi-cistronic vector encoding both murine SLPI (followed by an internal ribosomal entry site (IRES)) and green fluorescent protein (GFP), under the control of a 531 bp fraction of the MCMV promoter.

The Ad-elafin vector was originally constructed by Dr. Jean-Michel Sallenave in Professor Jack Gauldie's laboratory at McMaster University, Hamilton, Ontario, Canada (Sallenave *et al.*, 1998). Ad-lacZ was originally constructed by Dr. Christina Addison and colleagues in Professor Frank Graham's laboratory at McMaster University (Addison *et al.*, 1997). Ad-mSLPI was constructed in-house in our laboratory. Ad-dl70/3 was a kind gift from Professor Gauldie and Dr Mary Hitt (McMaster University). Ad-m-eotaxin was kindly provided by Professor Zhou Xing (McMaster University). Ad-IκB was a kind gift from Dr R. Balfour Sartor, University of North Carolina, NC, USA (Jobin *et al.*, 1998).

2.1.5. LPS and reagents related to LPS-binding studies

Escherichia coli O55:B5 LPS (smooth-form serotype) and polymyxin B sulphate were from Sigma.

Biotinylated *E. coli* O55:B5 LPS, recombinant human LPS-binding protein (LBP) and anti-LBP monoclonal antibody (mAb) HM2 were kind gifts from Dr. Anita Vreugdenhil (University of Maastricht, The Netherlands).

E. coli O6, O12, O15, O18 and *Pseudomonas aeruginosa* O2 LPS (smooth-form serotypes) and *E. coli* K12, R2 and R3 LPS (rough-form serotypes) were kind gifts from Dr. Richard Gibbs (Department of Medical Microbiology, University of Edinburgh, UK).

Human LBP peptide (1.8kDa), natural human LBP and monoclonal murine antibody to polymyxin B were obtained from HyCult Biotechnology, Uden, The Netherlands.

Human neutrophil elastase (HNE) was from Elastin Products, Owensville, MO, USA.

Recombinant human SLPI was from R & D Systems (Abingdon, UK).

2.1.6. Bacteria

P. aeruginosa strains PAO1 and J1385, and *Staphylococcus aureus* strain C1705 were available in-house, and were kindly provided by Professor John Govan, Department of Medical Microbiology, University of Edinburgh. Bacteria were stored at –70°C in 1ml of 10% w/v skim milk (Oxoid, Basingstoke, UK). After thawing, bacterial isolates were inoculated onto either *Pseudomonas* Isolation Agar (PIA) (45g/l agar base (Difco Laboratories, Detroit, MI, USA) plus 2% (v/v) glycerol) or Columbia agar (39g/l agar base (Oxoid)) and incubated overnight at 37°C. Bacteria were subcultured every 3-4 days thereafter. All media were prepared using distilled water and sterilised by autoclaving at 121°C at 15psi (pounds per square inch) for 15mins.

2.1.7. Mice

Male C57BL/6J and BALB/c mice aged between 6 and 8 weeks were obtained from Charles River UK, Margate, UK.

2.2. METHODS

2.2.1. TECHNIQUES RELATED TO ELAFIN/LPS BINDING STUDIES

2.2.1.1. LPS separation using Invitrogen NuPAGE (polyacrylamide gel electrophophoresis) Novex Bis-Tris electrophoresis system (minigels)

LPS samples were subjected to electrophoresis using the NuPAGE Novex Bis-Tris electrophoresis system (Invitrogen, Paisley, UK) according to the manufacturer's instructions. LPS was diluted to the appropriate concentration in dH_2O, and mixed with reducing sample buffer to a total sample volume of 20µl. Electrophoresis was performed at 200V for 35 minutes in SDS (sodium dodecyl sulphate)-free Bis-Tris-HCl buffered (pH 6.4) polyacrylamide gels with a concentration gradient of 4-12%, using denaturing SDS electrode buffer. Pre-stained markers were provided with the kit (Invitrogen). Bands were then detected using a Bio-Rad Silver Stain kit (Bio-Rad, Hemel Hempstead, UK).

2.2.1.2. LPS separation using maxigel PAGE (pH 8.8)

A maxigel PAGE technique was used to investigate the effects of varying the SDS content of electrode and sample buffers on the resolution of LPS banding. Samples of LPS were mixed 1:1 with sample buffer (± SDS) to a total volume of 10µl, and 25µg of each LPS serotype was separated. Electrophoresis was performed on SDS-free 14% gels at 60V-150V for 3 hours in a vertical maxigel electrophoresis tank, using Tris-Glycine electrode buffer ± SDS. LPS banding was detected by silver staining (Bio-Rad).

Formulations. *2x PAGE sample buffer*: 0.125M Tris-HCl, 20% (v/v) glycerol, ± 4% (w/v) SDS, 0.002% (v/v) bromophenol blue and 2% (v/v) mercaptoethanol. *Polyacrylamide gels*: Separating gel (pH 8.8) – 0.375M Tris-HCl, 14% (v/v) acrylamide (Life Technologies, Paisley, UK), 0.075% (v/v) ammonium persulphate (APS) and 0.14% (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED) (Bio-Rad). Stacking gel (pH 6.8) - 0.125M Tris-HCl, 4% (v/v) acrylamide, 0.075% (v/v) APS and 0.2% (v/v) TEMED. *Electrode buffer*: 0.025M Tris, 0.192M glycine, ± 0.1% (w/v) SDS.

2.2.1.3. Dot blotting

Briefly, 2μg of LPS or HNE were spotted onto Genescreen Plus nylon membrane (NEN Life Science Products, Boston, MA, USA) and allowed to dry. Alternatively, 25ng to 200ng of synthetic full-length elafin were spotted as positive controls. Synthetic full-length elafin from 25ng to 200ng was then dotted onto the LPS, HNE or onto an area to which no LPS or HNE had been added. The membrane was dried, and non-specific protein binding sites blocked for 1 hour at 4°C with PBS/0.1% Tween 20 containing 5% skim milk (Marvel, Wirral, UK). The membrane was then incubated in a 1:1000 dilution of rabbit anti-elafin IgG in blocking solution for 1 hour at room temperature (RT), and washed thoroughly with PBS/0.1% Tween. HRP-conjugated goat anti-rabbit immunoglobulin (Dako, Glostrup, Denmark) diluted 1:2000 in blocking solution was added for 30 minutes at RT, and then the membrane was washed as above. Finally, elafin was detected by enhanced chemiluminescence (ECL) (Amersham Pharmacia, Amersham, UK) as per manufacturer's instructions, and membrane developed on X-omat film (Kodak, Chalon, France).

2.2.1.4. PAGE (pH 8.8) (minigel) system to investigate binding of peptides to LPS

Interaction between peptides (full-length elafin, NH_2-terminal elafin domain, COOH-terminal elafin domain, SLPI and polymyxin B) and LPS was investigated in two variations of pH 8.8 PAGE. In these assays, sample buffer and gels were SDS-

free and non-denaturing, while SDS was included in electrode buffer only when stated specifically in figure legends. In experiments using completely SDS-free systems, peptides were incubated alone or with *E. coli* O15 LPS overnight at 37°C in PBS containing 1mM EDTA, and mixed with sample buffer to a total volume of 25µl prior to electrophoresis. In separate experiments where SDS was added to electrode buffer, peptides were incubated alone or with *E. coli* O55:B5 LPS for 30 minutes at 37°C in dH_2O, and mixed with sample buffer to a total volume of 25µl prior to electrophoresis. Differences between the assays (in terms of sample incubation period, reaction mixture and LPS serotype) are as a result of observations made during these studies concerning conditions required for binding, and availability of LPS.

Electrophoresis was performed on 15% gels at 70V-110V for 2-3 hours in a vertical electrophoresis tank Mini Protean II system (Bio-Rad). Bands were detected either by silver stain (Bio-Rad) or by immunoblotting. In the latter case, proteins were transferred onto Hybond ECL nitrocellulose membrane (Amersham Pharmacia) at 110V for 1 hour. Non-specific protein binding sites were blocked overnight at 4°C with PBS/0.1% Tween 20 containing 5% skim milk. Elafin was then detected with rabbit anti-elafin IgG, followed by HRP-conjugated goat anti-rabbit immunoglobulin, as described under 'Dot blotting' in section 2.2.1.3. above. Polymyxin B was detected with a murine anti-polymxin B monoclonal antibody, followed by goat anti-mouse immunoglobulin (Dako). Membranes were developed as per section 2.2.1.3.

Formulations. *4x PAGE sample buffer*: 0.5M Tris-HCl, 40% (v/v) glycerol, 0.2M EDTA and 0.5% (v/v) bromophenol blue. *Polyacrylamide gels*: Separating gel (pH 8.8) – 0.375M Tris-HCl, 2mM EDTA, 15% (v/v) acrylamide, 0.1% (v/v) APS and 0.05% (v/v) TEMED. Stacking gel (pH 6.8) – 0.125M Tris-HCl, 2mM EDTA, 4.5% (v/v) acrylamide, 0.1% (v/v) APS and 0.06% (v/v) TEMED. *Electrode buffer (± SDS)*: 0.6% (w/v) Tris base, 2.9% (w/v) glycine, 0.7% (w/v) EDTA, ± 0.1% (w/v) SDS. *Transfer buffer*: 24.7mM Tris base, 210mM glycine, and 20% (v/v) methanol.

2.2.1.5. Acidic (pH 4.5) native PAGE (minigels)

Peptides and LPS were diluted to the appropriate concentration in dH_2O, and incubated together for 30 minutes at 37°C. Where one peptide was incubated with LPS prior to the addition and incubation of a second peptide with reaction mixture, each incubation was performed for 30 minutes at 37°C. In all cases, final sample volume was 24µl. 6µl of sample buffer was added to each sample before electrophoresis. Electrophoresis was performed on 15% potassium hydroxide-acetic acid gels at 80V for 2-3 hours in a vertical electrophoresis tank Mini Protean II system, using a procedure modified from Reisfeld *et al.* (1962). Protein bands were detected by coomassie staining.

Formulations. *5x sample buffer*: 480mM potassium hydroxide, 40% (v/v) glycerol, 2.9% (v/v) glacial acetic acid, 0.5% (w/v) methyl green. *Polyacrylamide gels*: Separating gel (pH 4.5) – 240mM potassium hydroxide, 2.15% (v/v) glacial acetic acid, 0.5% (v/v) TEMED, 15% (v/v) acrylamide and 0.14% (v/v) APS. Stacking gel (pH 6.8) – 63mM potassium hydroxide, 0.38% (v/v) glacial acetic acid, 0.06% (v/v) TEMED, 2.5% (v/v) acrylamide and 0.15% (v/v) APS. *Electrode buffer*: 3.12% (w/v) β-alanine and 0.8% (v/v) glacial acetic acid. *Transfer buffer*: 24.7mM Tris base, 210mM glycine, and 20% (v/v) methanol.

2.2.1.6. Coomassie staining

Gels were incubated for 30 minutes at room temperature in fixative (10% (v/v) glacial acetic acid and 20% (v/v) methanol), followed by 30 minute incubation in coomassie stain (0.25% (w/v) coomassie blue, 7.5% (v/v) glacial acetic acid and 50% (v/v) methanol). Gels were then destained overnight at RT in destaining solution (7% (v/v) glacial acetic acid and 10% methanol).

2.2.1.7. ELISA technique to investigate inhibition of the LPS-LBP interaction

This technique measures the binding of biotinylated LPS to immobilised LBP. The method was carried out according to Scott *et al.* (2000b). Briefly, the anti-LBP mAb HM21 was diluted to 10mg/ml in PBS and adsorbed onto a 96-well assay plate (Corning Costar, High Wycombe, UK) overnight at 4°C. The plate was then blocked with PBS containing 1% bovine serum albumin (BSA) (Sigma) for 1 hour at RT and washed with 0.1% Tween 20 in dH_2O. Recombinant LBP (25ng/ml) diluted in PBS/0.1% BSA was added to the plate for 1.5 hours at RT. Following washing, biotinylated *E. coli* O55:B5 LPS was added in the presence or absence of peptides. The peptides were pre-incubated with biotinylated LPS for 30 minutes at 37°C in PBS prior to addition to the wells. Following 1hour incubation at RT and washing, binding of biotinylated LPS to immobilised LBP was detected using horseradish peroxidase (HRP)-conjugated streptavidin (Dako) diluted 1:2000 in PBS/0.1% BSA. The plate was incubated for 1 hour at RT, washed, and 3,3',5'5-tetramethylbenzidine (TMB) (Boehringer Mannheim UK, Lewes, UK) added 1:50 in H_2O_2/sodium acetate citrate (pH 4.9) for 15 minutes. The reaction was stopped by addition of 1M H_2SO_4 (BDH, Poole, UK) and the O.D. read at 490nm (MRX II microplate reader, Dynex Technologies, Billinghurst, UK).

2.2.1.8. ELISA technique to investigate elafin/LBP competition for binding to LPS

An ELISA technique was devised to measure binding of elafin to immobilised LPS. LPS alone binds poorly to assay plates, therefore LPS was pre-bound to polymyxin B to form complexes which are stable when coated onto microplates; this method was adapted from Scott and Barclay (1987). Briefly, equal volumes of 36μg/ml *E. coli* O55:B5 LPS and 20μM polymyxin B (both diluted in dH_2O) were mixed with continuous shaking for 30 minutes at room temperature. The mixture was then dialysed overnight against dH_2O using a 3.5kDa pore size, in order to remove excess unbound polymyxin B.

50µl/well of this mixture was coated onto a 96-well assay plate overnight at 4°C. The plate was then blocked with PBS containing 1% BSA for 1 hour at RT and washed with 0.1% Tween 20 in dH_2O. 156nM full-length elafin was added for 1.5 hours at RT. Alternatively, 25ng/ml LBP was added to wells simultaneously with elafin, or LBP was added 45 minutes after addition of elafin and then incubated for a further 45 minutes. Following washing, rabbit anti-elafin IgG diluted 1:1000 in PBS/0.1% BSA was added and incubated for 1 hour at RT. The plate was washed, then incubated for 1 hour at RT with HRP-conjugated goat anti-rabbit immunoglobulin diluted 1:2000 in PBS/0.1% BSA. Following washing, the plate was developed and read as described previously in section 2.2.1.7.

2.2.2. CELL CULTURE

The RAW 264.7 murine macrophage cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 0-10% (v/v) foetal calf serum (FCS; heat-inactivated at 57°C for 1 hour prior to use) (Labtech, Ringmer, UK), 100 U/ml penicillin (Gibco BRL, Paisley, UK), 100µg/ml streptomycin (Gibco), 4mM L-glutamine (Gibco), 4.5g/l glucose and 1.5g/l sodium bicarbonate. For all following methods involving use of the RAW 264.7 macrophage cell line, 'DMEM' is used to describe this culture medium with no FCS added, unless otherwise indicated.

The murine Clara (bronchial epithelial) cell line DJS2-2 was a kind gift from Dr Franco DeMayo (Baylor College of Medicine, Houston, TX, USA). Cells were cultured in DMEM supplemented with 0-10% (v/v) FCS, 100 U/ml penicillin, 100µg/ml streptomycin, and 4mM L-glutamine. For all following methods involving the use of Clara cells, 'DMEM' is used to describe this culture medium with no FCS added, unless otherwise indicated.

All cells were maintained at 37°C in a humidified incubator containing 5% CO_2, and used for assays at the lowest possible passage number. Estimation of the

number of cells in each monolayer was performed by washing cells with phosphate buffered saline (PBS) (Sigma), incubating with trypsin (Gibco) at 37°C to facilitate detachment, and counting of cells using a haemocytometer (Sigma). Cell viability was assessed by staining with trypan blue (Sigma), with exclusion indicating viability.

2.2.3. ELAFIN PEPTIDE/LPS CELL-BASED ASSAYS

2.2.3.1. Stimulation of RAW 264.7 murine macrophages with LPS

Macrophages were seeded at 5 x 10^5 cells/well of a 48-well plate (Corning Costar) and incubated overnight at 37°C. Cells were washed twice with PBS and 500µl of fresh DMEM added; depending on the experiment, DMEM contained varying concentrations of FCS between 0 and 10% (v/v), and in one assay was replaced with 25ng/ml natural human LBP (approximately equivalent to the concentration of LBP present in 0.5% serum). *E. coli* O55:B5 LPS in the concentration range 0 to 5µg/ml was incubated with cells for 4 hours at 37°C, and supernatant retrieved.

2.2.3.2. Investigation of the effects of elafin peptides on the TNF-α response of RAW 264.7 murine macrophages to LPS

Macrophages were seeded at 5 x 10^5 cells/well of a 48-well plate (Corning Costar) and incubated overnight at 37°C. Cells were washed twice with PBS, and 500µl of either serum-free DMEM or DMEM containing 0.2% serum added. 50ng/ml *E. coli* O55:B5 LPS was incubated with cells for 4 hours at 37°C either alone or in the presence of elafin peptides. In the latter case, 2.5µl samples of LPS were either incubated with 5µl samples of elafin peptides for 30 minutes at 37°C prior to addition to cells, or LPS and elafin peptides were added to cells simultaneously. Total sample volume was 7.5µl, and both LPS and elafin were diluted to test concentrations in dH_2O. When cells were stimulated with LPS in the absence of elafin, 2.5µl LPS was added with 5µl dH_2O. In alternative experiments

designed to investigate the effects on TNF-α secretion of elafin peptides alone, peptides were incubated with cells for 4 hours at 37°C as above. In all cases, cell supernatants were analysed by TNF-α ELISA using a commercial ELISA kit (Duoset, R & D Systems) in accordance with the manufacturer's instructions. The standard curve measured between 15.6pg/ml and 1000pg/ml protein, and test samples were diluted accordingly in PBS containing 1% BSA.

2.2.3.3. Investigation of the effects of elafin peptides on the MIP-2 response of murine Clara cells to LPS

Clara cells were seeded at 3 x 10^5 cells/well of a 48-well plate and incubated overnight at 37°C. Cells were washed twice with PBS, and 500μl of either serum-free DMEM or DMEM containing 0.2% serum added. 50ng/ml (in medium containing serum) or 1μg/ml (in serum-free medium) *E. coli* O55:B5 LPS was incubated with cells for 4 hours at 37°C either alone or in the presence of elafin peptides. In the latter case, samples of LPS were either incubated with 5μl samples of elafin peptides for 30 minutes at 37°C prior to addition to cells, or LPS and elafin peptides were added to cells simultaneously. 50ng/ml LPS was made up in 2.5μl samples, while 1μg/ml LPS was made up in 5μl. Total sample volume was therefore 7.5μl or 10μl, and both LPS and elafin were diluted to test concentrations in dH_2O. When cells were stimulated with LPS in the absence of elafin, LPS was added with 5μl dH_2O. In alternative experiments designed to investigate the effects on MIP-2 secretion of elafin peptides alone, peptides were incubated with cells for 4 hours at 37°C as above. In all cases, cell supernatants were analysed by MIP-2 ELISA using a commercial ELISA kit (Duoset, R & D Systems) in accordance with the manufacturer's instructions. The standard curve measured between 15.6pg/ml and 1000pg/ml protein, and test samples were diluted accordingly in PBS containing 1% BSA.

2.2.4. ADENOVIRUS/LPS CELL-BASED ASSAYS, & RELATED TECHNIQUES

2.2.4.1. Infection of RAW 264.7 murine macrophages with adenoviral constructs (Ad-elafin, Ad-lacZ, Ad-IκB, Ad-dl70/3, Ad-m-eotaxin, Ad-mSLPI or vehicle alone)

Macrophages were seeded at 5 x 10^5 cells/well of a 48-well plate and incubated overnight at 37°C. Adenoviral constructs of known concentration were diluted in Minimum Essential Eagle's Medium (MEEM) (see Formulations below) with or without the addition of $CaCl_2$ (to form calcium phosphate (CaPi) precipitates), or in DMEM, and incubated for 20 minutes at room temperature to allow formation of Ad:CaPi coprecipitates where applicable (Fasbender *et al.*, 1998). Adenovirus was applied to cells at various multiplicities of infection (mois) in a total volume of 250µl for 45 minutes at 37°C (macrophages were infected at moi 100 for the purpose of the LPS stimulation assays described in Chapter 5). Cells were washed twice with PBS, and incubated overnight at 37°C in DMEM containing 0.2% FCS.

Formulations. *MEEM: (M2279; Sigma)*. This medium contains 1.8mM Ca^{2+} and 0.86mM Pi. Addition of $CaCl_2$ to this medium facilitates formation of CaPi precipitates; for example, addition of 4mM $CaCl_2$ allows formation of precipitates with a total of 5.8mM Ca^{2+} and 0.86mM Pi.

2.2.4.2. Investigation of the effects of calcium phosphate (CaPi) precipitates on the inflammatory responses of RAW 264.7 murine macrophages

Macrophages were seeded at 5 x 10^5 cells/well of a 48-well plate and incubated overnight at 37°C. Cells were then incubated for 45 minutes at 37°C with either 250µl DMEM or 250µl MEEM containing 4mM $CaCl_2$ (forming CaPi precipitates with 5.8 mM Ca^{2+} and 0.86 mM Pi, as described in 'Formulations' in section 2.2.4.1.); these media were incubated at room temperature for 20 minutes prior to addition to cells. Cells were washed twice with PBS and 500µl DMEM containing 0.2% FCS, added. Cells were then incubated at 37°C for 1hour or 23

hours, and media analysed by TNF-α ELISA. Alternatively, following 23-hour incubation, cells were stimulated with 50ng/ml *E. coli* O55:B55 LPS for 4 hours at 37°C, and media analysed by TNF-α ELISA. Cell viability was also assessed by light microscopy, trypan blue exclusion and cell counting.

2.2.4.3. Stimulation of adenovirally-infected RAW 264.7 murine macrophages with LPS

Macrophages were infected with adenovirus constructs as described in section 2.2.4.1., and incubated overnight at 37°C in DMEM containing 0.2% FCS. For experiments investigating the effects of LPS on elafin production by Ad-elafin-infected macrophages, cells were incubated for 4 hours at 37°C with concentrations of *E. coli* O55:B5 LPS ranging between 0 and 5μg/ml; cell supernatants or lysates were then analysed by elafin ELISA. For experiments investigating the LPS-responsiveness of Ad-infected macrophages, cells were incubated for 4 hours at 37°C with 50ng/ml *E. coli* O55:B5 LPS, either in conditioned media or following washing with PBS and addition of fresh DMEM containing 0.2% FCS. Alternatively, conditioned media were removed from Ad-infected cells 4-hours or 23-hours post-viral infection, media added to 5 x 10^5 uninfected, washed cells and then these cells were incubated for 4 hours with 50ng/ml LPS as above. Cell supernatants were analysed by TNF-α ELISA.

2.2.4.4. Staining for β-galactosidase in RAW 264.7 murine macrophages

Macrophages, infected with Ad-lacZ as described in section 2.2.4.1., were fixed for 10 minutes at room temperature in a solution comprising 0.2% glutaraldehyde, 0.8% formaldehyde and 2mM $MgCl_2$ in PBS. Fixative was discarded and 200μl of staining solution added for 5 hours at 37°C (staining solution comprised 5mM $K_4Fe(CN)_6$, 5mM $K_3Fe_3(CN)_6$, 2mM $MgCl_2$, 0.05% Triton X-100, 0.5mg/ml X-gal, in PBS). Cells were washed with PBS to remove residual stain, air-dried and photographed.

2.2.4.5. Lysis of cells for measurement of intracellular elafin

Ad-elafin-infected cells were washed once with cold PBS and lysed using a buffer containing 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate and 1% (w/v) SDS in PBS. One protease inhibitor tablet (Boehringer Mannheim) was added per 10ml of lysis buffer. Cells were scraped into an eppendorf, incubated on ice for 30 minutes and then centrifuged at 13000rpm for 20 minutes at 4°C. Supernatants (representing total cell lysates) were removed and stored at -40°C until analysis by elafin ELISA, as described in section 2.2.4.6. below.

2.2.4.6. Elafin ELISA

Concentrations of human elafin in supernatants and lysates of Ad-elafin-infected cells were determined using a sandwich ELISA available in-house. This technique has previously been described in detail by Reid *et al.* (1999). Samples were diluted according to the range of the standard curve (0.5-10ng/ml).

2.2.5. ANTIMICROBIAL STUDIES & RELATED TECHNIQUES

2.2.5.1. Infection of murine Clara cells with adenoviral constructs (Ad-elafin, Ad-lacZ or vehicle alone) and investigation of antimicrobial activity against *P. aeruginosa*

Clara cells were grown to confluence in 96-well plates in DMEM containing 10% FCS, and then incubated for 45 minutes at 37°C with adenoviral vectors (moi 100) in a total volume of 100µl DMEM/ 10% FCS. Cells were washed twice with PBS, and incubated for 2-6 days at 37°C in 100µl DMEM/ 10% FCS lacking penicillin and streptomycin. *P. aeruginosa* strain PAO1 was inoculated into 10ml nutrient broth (Oxoid) containing 5% yeast extract (Difco) and incubated overnight at 37°C in an orbital shaker at 200 rev/min (Gallenkamp, Fisher Scientific, Loughborough, UK). The culture was centrifuged at 4500rpm for 10 minutes at room temperature (Biofuge, Heraeus Instruments, Kendro, Bishops Stortford, UK), supernatant discarded and the pellet resuspended in 8 ml phosphate buffer (0.008M

K_2HPO_4/ 0.002M KH_2PO_4, pH 7.4). The suspension was adjusted with phosphate buffer to an absorbance of 1.45 read at 590nm (M330 UV-visible spectrophotometer, Camspec, Cambridge, UK), yielding an estimated bacterial concentration of 2.2 x 10^{11} colony forming units (cfu)/ml. Serial dilutions of this suspension were made in DMEM/ 10% FCS lacking penicillin and streptomycin, and 100µl aliquots were incubated with cells for 5 hours at 37°C. Following incubation, media were serially diluted and 100µl of samples plated onto PIA. Plates were incubated for 16 hours at 37°C and colonies counted. Remaining cell culture media were retained and frozen at -20°C.

2.2.5.2. SDS-PAGE & Western Blotting to detect elafin in culture media from Ad-elafin-infected Clara cells

Culture media from the antimicrobial assays described in section 2.2.5.1. were analysed to detect secreted elafin. Briefly, 22.5µl samples of media were resolved on 15% SDS-polyacrylamide gels using a vertical electrophoresis tank Mini Protean II system. Samples were electrophoresed at 70V-110V using SDS-Tris-Glycine electrode buffer for 2-3 hours beside pre-stained molecular weight markers (Life Technologies), and using 40ng of synthetic full-length elafin as a control.

Following separation, proteins were transferred onto Hybond ECL nitrocellulose membrane at 110V for 1 hour. Non-specific protein binding sites were blocked overnight at 4°C with PBS/0.1% Tween 20 containing 5% skim milk. The membrane was then incubated in a 1:1000 dilution of rabbit anti-elafin IgG in blocking solution for 1 hour at room temperature, and washed thoroughly with PBS/0.1% Tween. HRP-conjugated goat anti-rabbit immunoglobulin diluted 1:2000 in blocking solution was added for 30 minutes at room temperature, and then the membrane was washed as above. Finally, immunoreactive bands were detected by ECL, and membrane developed on X-omat film.

Formulations. *4x SDS-PAGE sample buffer*: 0.5M Tris-HCl, 40% (v/v) glycerol, 8% (w/v) SDS, 0.2M EDTA, 0.5% (v/v) bromophenol blue and 4% (v/v)

mercaptoethanol. *SDS-polyacrylamide gels*: Separating gel (pH 8.8) – 0.375M Tris-HCl, 2mM EDTA, 0.1% (w/v) SDS, 15% (v/v) acrylamide, 0.1% (v/v) APS and 0.05% (v/v) TEMED. Stacking gel (pH 6.8) – 0.125M Tris-HCl, 2mM EDTA, 0.1% (w/v) SDS, 4.5% (v/v) acrylamide, 0.1% (v/v) APS and 0.06% (v/v) TEMED. *Electrode buffer*: 0.6% (w/v) Tris base, 2.9% (w/v) glycine, 0.7% (w/v) EDTA, 0.1% (w/v) SDS. *Transfer buffer*: 24.7mM Tris base, 210mM glycine, and 20% (v/v) methanol.

2.2.5.3. Isolation and culture of primary murine tracheal epithelial cells

All work complied with the UK Animal (Scientific Procedures) Act 1986. The method used followed approximately that described in the paper by Davidson *et al.* (2000). Mice were killed by asphyxiation with CO_2, and doused with 70% ethanol. Tracheae were excised and severed at the proximal surface of the thyroid cartilage and at the junction of the bronchi. The thyroid glands and other adherent tissue were removed before cutting tracheae lengthways, rinsing in PBS and transferring to collection medium at 37°C (see Formulations). Batches of eight tracheae were removed to 20ml aliquots of dissociation medium containing DNase and Pronase (see Formulations) and incubated for 60 mins at 37°C. 5ml of FCS was added to halt enzymatic activity, and samples carefully inverted 12 times each to dissociate epithelial cells. Tracheae were transferred to 10ml of fresh culture medium (see Formulations) and gently agitated a further 12 times to facilitate additional release of epithelial cells. The resultant cell suspensions were pooled and centrifuged at 1300 rpm for 6 mins at 24°C, supernatants removed and pellets resuspended in 10ml culture medium. Samples were again centrifuged at 1300rpm for 6 mins at 24°C, and pellets resuspended in 5ml of culture medium. Cell suspensions were incubated at 37°C for 2 hours in 10cm culture dishes (Premaria, Becton Dickinson UK, Oxford, UK) to remove adherent non-epithelial cells. Media were removed to collect unattached cells, centrifuged at 1300rpm for 6 mins, and resuspended in 200µl culture medium per two tracheae. Dissociated cells from two tracheae (approximately 4 x 10^5 cells) were seeded onto one tissue culture insert semi-permeable support membrane (Costar Transwell clear, tissue culture-treated

polyester membrane 24-well plate inserts, 0.4μm pore; Corning Costar) in 200μl of culture medium, with 600μl outside the insert. Inserts were pre-coated with 100μl of type VI human placental collagen solution (0.5mg/ml collagen in dH_2O with 0.2% glacial acetic acid), air-dried overnight, and washed twice with PBS before use. Cells were incubated at 37°C in 6% CO_2 for 3 days. On day 4, medium bathing the apical cell surface was removed along with any cell debris, and the medium on the outside of the insert replaced with 600μl of Ultroser G (USG) medium (see Formulations). Medium bathing the basolateral surface was replaced twice weekly.

Formulations. *Collection medium*: 1:1 mix of DMEM and Ham's F-12 medium (Gibco) containing 100 U/ml penicillin and 100μg/ml streptomycin. *Dissociation medium*: calcium- and magnesium-free MEEM containing 60 U/ml penicillin, 60μg/ml streptomycin, 1.4mg/ml Pronase (Boehringer Mannheim) and 0.1mg/ml DNase (Sigma). *Culture medium*: collection medium containing 5% FCS and 120 U/l insulin (Nova Human Actrapid, AAH Pharmaceuticals, Glasgow, UK). *USG medium*: collection medium containing 2% USG serum substitute (Gibco).

2.2.5.4. Infection of primary murine tracheal epithelium with adenoviral constructs (Ad-elafin, Ad-lacZ or vehicle alone)

Adenoviral constructs of known concentration were diluted in MEEM with or without the addition of 4mM $CaCl_2$ (to form CaPi precipitates), or in USG medium, and incubated for 20 minutes at room temperature to allow formation of Ad:CaPi coprecipitates where applicable (Fasbender *et al.*, 1998). Adenovirus was applied to epithelial cells at an moi of 100 in a total volume of 250μl for 45 minutes at 37°C. Cells were washed twice with PBS, and incubated for 18-20 hours at 37°C in the absence of media bathing the apical surface.

2.2.5.5. Staining for β-galactosidase in primary murine tracheal epithelium

Epithelial cells, infected with Ad-lacZ as described in section 2.2.5.4., were stained for β-galactosidase production according to the same technique used to stain Ad-lacZ-infected RAW 264.7 murine macrophages in section 2.2.4.4. Similarly, cells were left to air dry and then photographed following staining.

2.2.5.6. Assay to investigate the effects of elafin gene augmentation on the antimicrobial activity of primary murine tracheal epithelium against *Pseudomonas aeruginosa* and *Staphylococcus aureus*

Colonies of *P. aeruginosa* strains PAO1 or J1385, or *S. aureus* strain C1705, were inoculated into 10ml nutrient broth containing 5% yeast extract and incubated overnight at 37°C in an orbital shaker at 200 rev/min. Bacterial suspensions were centrifuged at 3000rpm for 15 minutes at room temperature, supernatant discarded and the pellet resuspended in 10ml phosphate buffer. Suspensions were adjusted with phosphate buffer to an absorbance of 1.00 read at 590nm, yielding an estimated bacterial concentration of 1×10^9 cfu/ml.

Serial dilutions of this suspension were performed in phosphate buffer down to 1×10^7 cfu/ml, and 20 nanolitres (nl) were added to cells using a Hamilton precision syringe (Hamilton Company, Reno, NV, USA) to provide an initial bacterial colony count of approximately 200. Concurrently, 20nl of bacterial suspension was plated onto several agar plates, along with 100µl phosphate buffer, to obtain more accurate values pertaining to the initial number of bacteria added to cells. Bacteria were incubated with cells for 3 hours at 37°C.

Cell inserts were washed with both 105µl phosphate buffer and 105µl phosphate buffer containing 0.5% Triton X-100 (to ensure removal of bacteria attached to cell surface). These samples were pooled and centrifuged for 10 minutes at 8000rpm; supernatants were removed for analysis by elafin ELISA, while bacterial pellets were resuspended in phosphate buffer and plated onto PIA (*P. aeruginosa*) or

Columbia agar (*S. aureus*), either neat or following serial dilution in phosphate buffer. Plates were incubated for 16 hours at 37°C and colonies counted.

2.2.6. STATISTICAL ANALYSIS

Results are reported either (i) as pooled data from a series of n separate experiments, each carried out in triplicate, and presented as mean ± S.E., or (ii) as individual experiments, carried out in triplicate, and presented either as mean ± S.E. or mean ± SD. Statistical significance was analysed by one-way analysis of variance with comparisons between groups made using the Bonferroni Multiple Comparison Test.

Statistical significance was assigned to data returning a P value of less than 0.05.

CHAPTER 3

INTERACTIONS OF ELAFIN WITH BACTERIAL LIPOPOLYSACCHARIDE

3.1. AIMS

The principal aim of the work described in this chapter was to determine whether elafin is capable of direct interaction with the lipopolysaccharide component of the outer membrane of Gram-negative bacteria. A secondary aim was to determine the relative contributions of the distinct structural domains of elafin to LPS-binding. Moreover, experiments were designed to investigate which region of the LPS molecule elafin may bind to.

In the event that an interaction between these molecules was observed, a further aim was to investigate how the binding of elafin to LPS would affect the interaction of LPS with LPS-binding protein, and to assess the relative affinity of these molecules for LPS.

The synthetic elafin molecules used in these experiments have been described in Chapter 2 (section 2.1.2.). In brief, these peptides are full-length elafin (approximately 9.9kDa), the NH_2-terminal domain (approximately 5.2kDa) and the COOH-terminal domain (approximately 4.8kDa).

3.2. RESULTS

3.2.1. Preliminary experiments to visualise LPS serotype ladders and establish conditions for initial interaction assays

As discussed in Chapter 1, LPS consists of three major portions, of which lipid A is considered the most important since this domain harbours the endotoxic properties of the molecule. This domain is also known to be the most conserved across Gram-negative bacterial species, and is recognised as an important binding

site for anti-endotoxin molecules (Morrison and Jacobs, 1976; Gazzano-Santoro *et al.*, 1992; Appelmelk *et al.*, 1994).

Four LPS serotypes of the *Escherichia coli* species were subjected to Bis-Tris polyacrylamide gel electrophoresis (PAGE) analysis and visualised by silver staining (Figure 1). *E. coli* O55:B5 LPS is a smooth-form LPS (indicated by the O prefix before the serotype number); this can be visualised by the presence of a number of bands detected above 6kDa in a characteristic 'ladder' pattern. K12, R2 and R3 are rough-form serotypes and therefore contain no oligosaccharide repeats. The lipid A-core portion of these rough serotypes is represented by a single band just below 6kDa. The position of the cathode and anode is indicated by encircled – or + signs next to all gels in this chapter.

In order to asses interaction of elafin with LPS using PAGE techniques, preliminary experiments were performed to assess the effects of SDS (sodium dodecyl sulphate) on the migration of LPS; it should be noted that the electrophoresis system used to separate LPS samples in Figure 1 contained SDS in electrode buffer, and the similar compound LDS (lithium dodecyl sulphate) in sample buffer. SDS (and indeed LDS) is an anionic detergent that is used in PAGE techniques to bind to and denature molecules, conferring to them an overall negative charge proportional to their size and, as such, can dissociate multimeric molecules (such as LPS) into their subunits. Thus SDS-PAGE allows molecules to be resolved by molecular weight, following migration to the anode. It was therefore hypothesised that SDS may be a hindrance to any potential interaction between cationic elafin molecules and anionic LPS molecules in subsequent experiments, hence every effort was made to minimise the presence of SDS throughout experimental conditions.

Figure 2 demonstrates the importance of the inclusion of SDS in electrode buffers to the separation of LPS into its component moieties. Firstly it must be noted that gels themselves contained no SDS. Absence of SDS in sample buffer effected only a minor disruption of LPS banding when SDS was added to electrode buffer (Figure 2A); however, exclusion of SDS from electrode buffers allowed LPS to

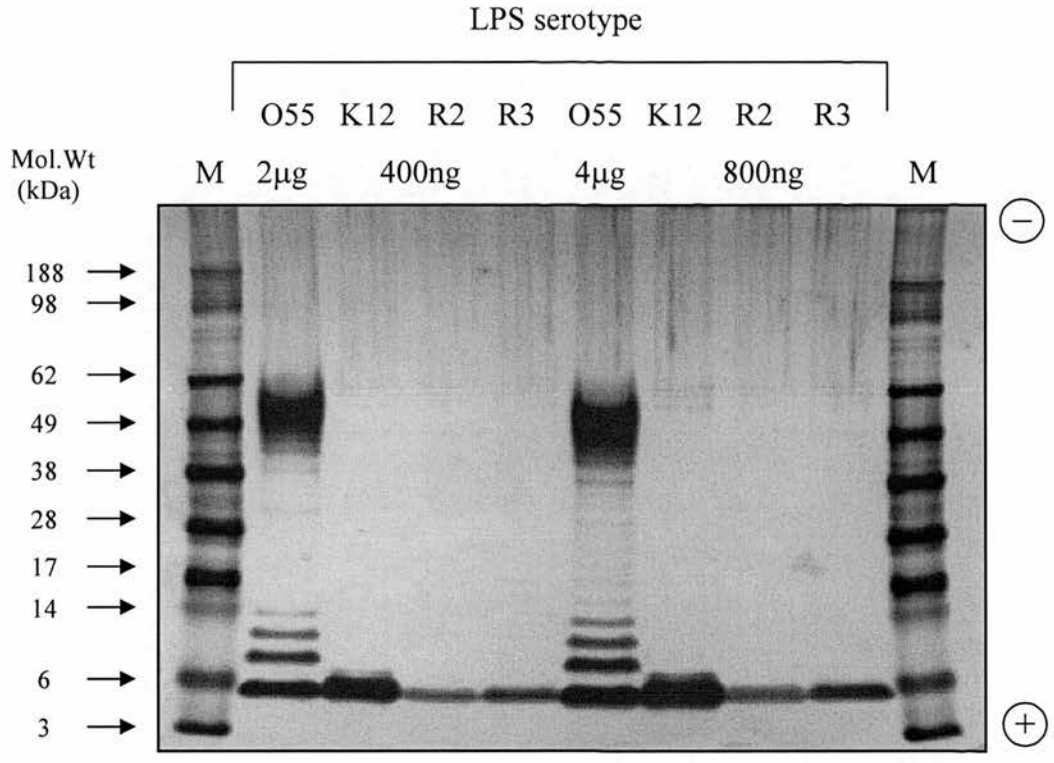


Figure 1. LPS serotypes separated as characteristic 'ladder' pattern

LPS samples were diluted to the appropriate concentration in dH_2O and mixed with reducing sample buffer to a total sample volume of 20μl. Electrophoresis was performed at 200V for 35 minutes in SDS-free Bis-Tris-HCl buffered (pH 6.4) polyacrylamide gels with a concentration gradient of 4-12%, using denaturing SDS electrode buffer; sample buffer contained LDS (lithium dodecyl sulphate) (Invitrogen NuPAGE Novex Bis-Tris electrophoresis system). Bands were visualised using the Bio-Rad Silver Stain Kit. M= molecular weight markers.

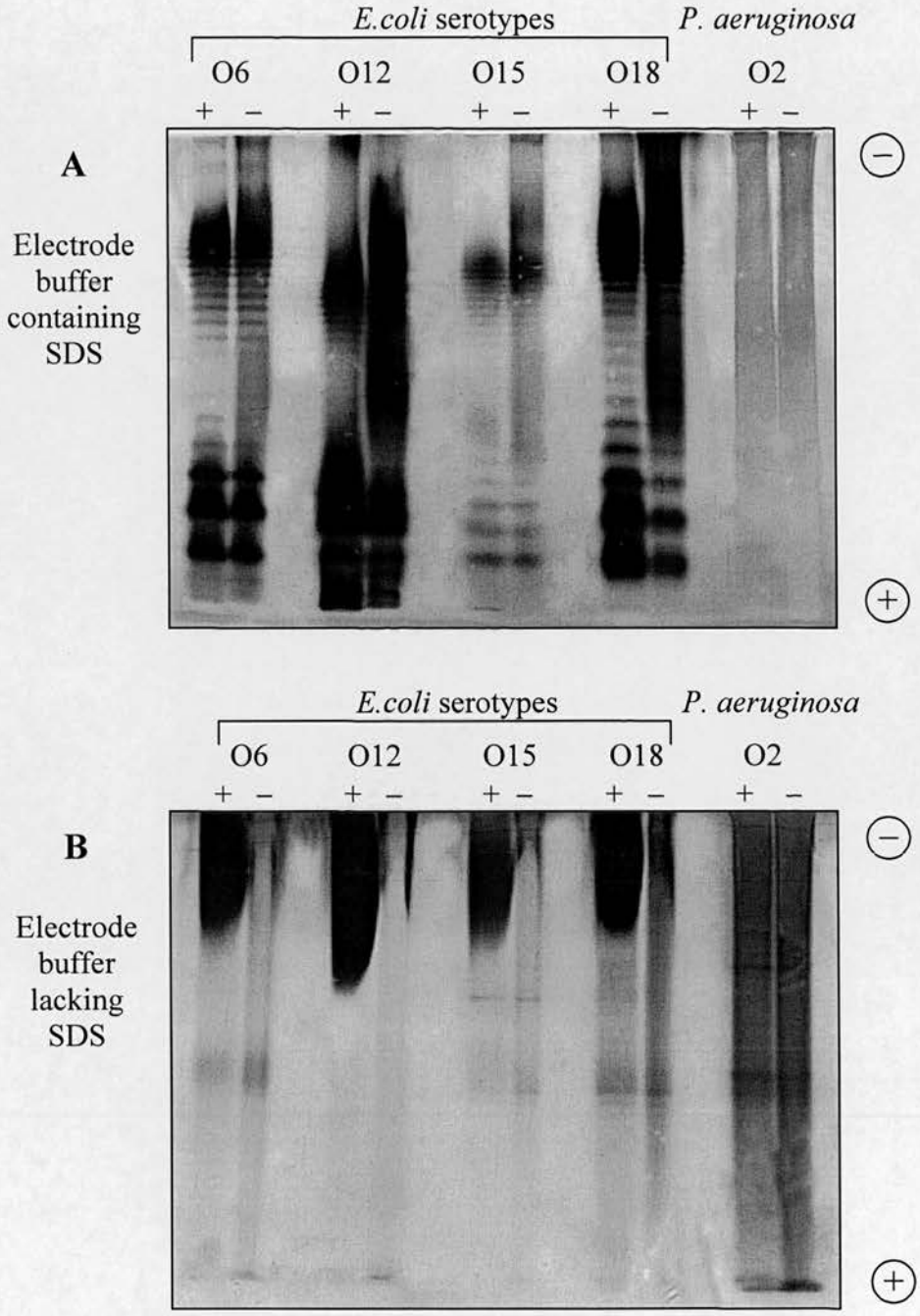


Figure 2. SDS in electrode buffer is critical for the resolution of LPS structure by PAGE

LPS samples were diluted to the appropriate concentration in dH_2O and mixed with sample buffer to a total volume of 10µl; each lane contains 25µg of LPS. Electrophoresis was performed in SDS-free 14% gels at 60V-150V for 3 hours using a pH 8.8 maxigel PAGE technique as detailed in 'Materials and Methods', and gels were silver stained. Sample buffer either contained or lacked SDS as per labels (+ = sample buffer containing SDS, – = sample buffer containing no SDS). Gel A was performed using electrode buffer containing SDS; gel B was performed using electrode buffer lacking SDS.

migrate only as a 'streak', whilst the absence of SDS in sample buffer further hindered the ability of the LPS molecules to migrate (Figure 2B).

3.2.2. Direct interactions of elafin with LPS

Although the inclusion of SDS in electrode buffer was shown to be essential in order to dissociate LPS into its 'ladder' pattern by PAGE, in the complete absence of SDS in any buffers LPS could still migrate weakly in an unresolved form, due to anionic charges on the molecules (Figure 2B). Initial experiments were therefore undertaken to investigate the interaction of elafin and elafin fragments with LPS using this native pH 8.8 PAGE technique, entirely devoid of SDS. Similar native PAGE techniques have been used by others to demonstrate binding of molecules such as sCD14 and SLPI to LPS (Hailman *et al.*, 1994; Ding *et al.*, 1999). Since previous work has described the LPS-binding properties of SLPI (Ding *et al.*, 1999), the other member of the antileukoprotease superfamily of proteinase inhibitors and a molecule with which elafin shares approximately 40% sequence homology (Wiedow *et al.*, 1990; Sallenave and Ryle, 1991; Sallenave and Silva, 1993), it seemed reasonable to use human SLPI as a control for binding to LPS. *E.coli* O15 LPS, as an example of a typical smooth-form LPS serotype, was used for the purpose of these investigations.

Full-length elafin was shown to inhibit the migration of LPS in this gel system, an observation that is suggestive of a direct interaction between the molecules (Figure 3). A similar observation was noted in the case of SLPI. For the purpose of these investigations the molecular weights of elafin and SLPI, 9.9kDa and 11.7kDa respectively, were considered to be similar enough to compare binding using 'exact' weights i.e. 250ng-5μg in this case. It must be noted that in these conditions neither elafin nor SLPI alone entered the gel matrix, presumably due to their net positive charge (lanes 2); Figure 4 demonstrates the same finding in the case of both the NH_2- and COOH-termini of elafin. In normal circumstances LPS migrates to the anode, as shown in lane 1 of all gels in Figures 3 and 4. Incubation with elafin or SLPI inhibited the migration of LPS, and this inhibition occurred at a lower ratio of elafin:LPS than SLPI:LPS. As the concentration of each proteinase

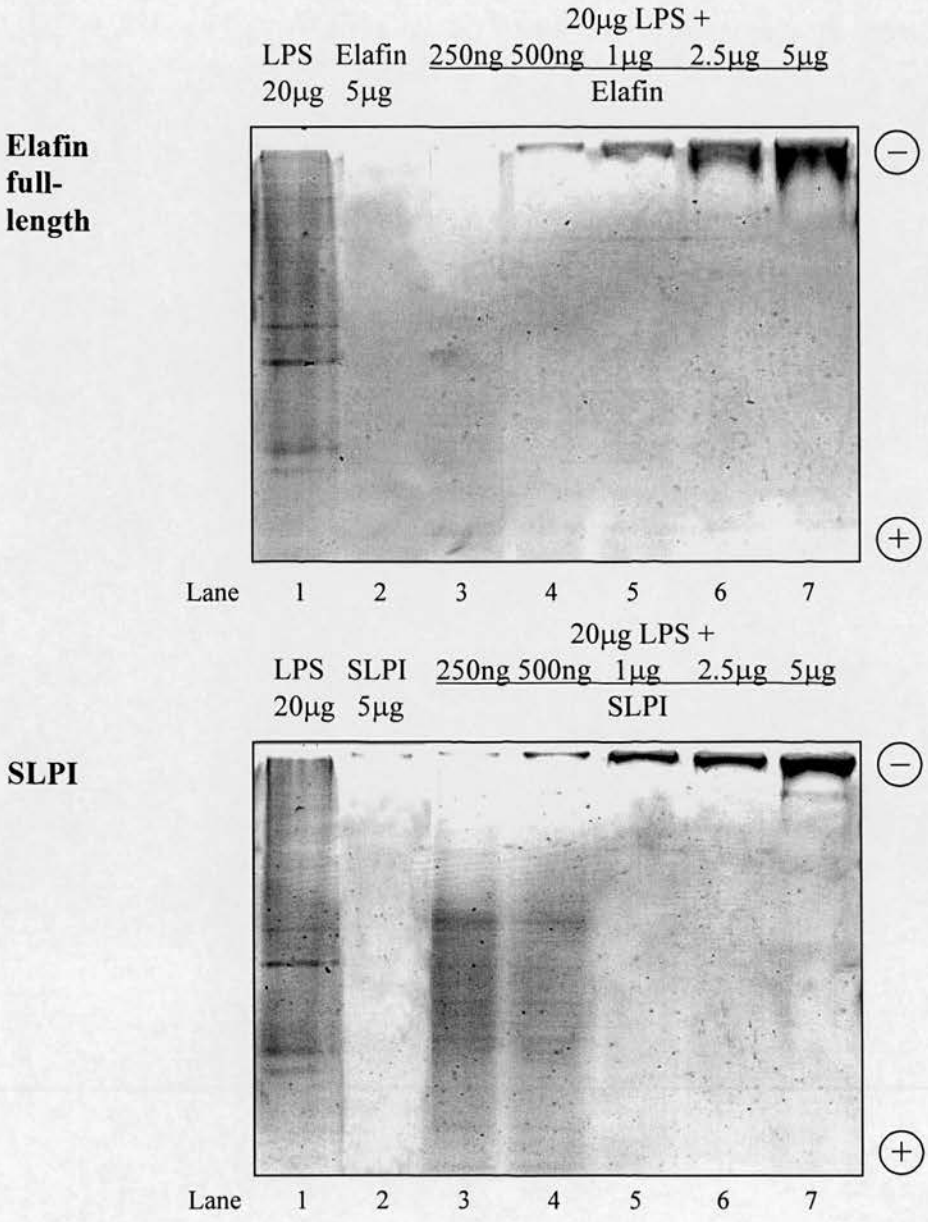


Figure 3. Elafin and SLPI inhibit migration of LPS in a pH 8.8 native gel system

Nondenaturing PAGE followed by silver staining. Samples of elafin or SLPI were incubated with *E.coli* O15 LPS at 37°C overnight in PBS containing 1mM EDTA, and mixed with SDS- and mercaptoethanol-free sample buffer to a total volume of 25μl prior to electrophoresis. Electrophoresis was performed in SDS-free 15% gels at 70V-110V for 2-3 hours, using pH 8.8 minigels as detailed in 'Materials and Methods'. Electrode buffer was SDS-free. Gels were silver stained.

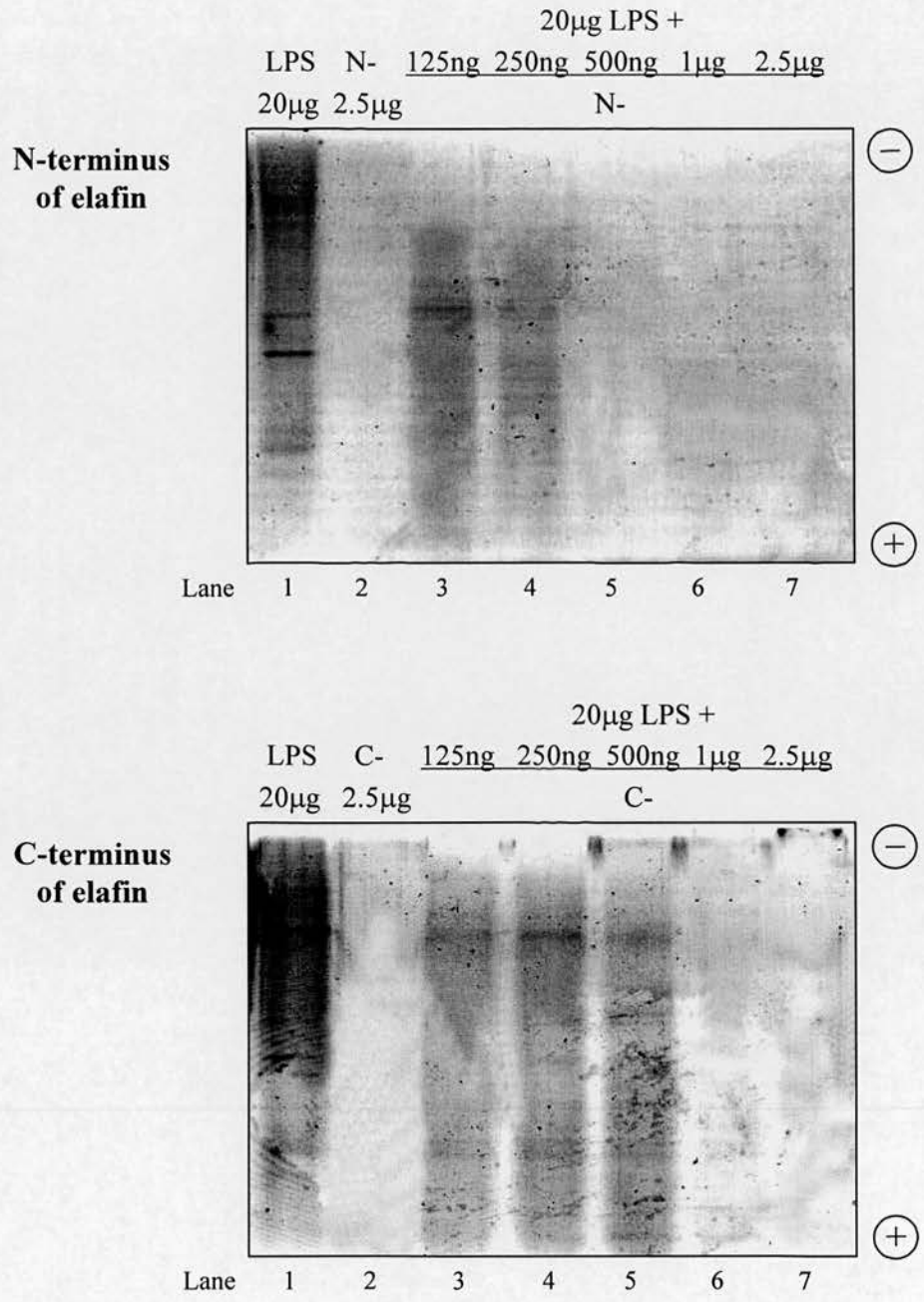


Figure 4. Elafin N-terminal and C-terminal domains inhibit migration of LPS in a pH 8.8 native gel system

Nondenaturing PAGE followed by silver staining. Samples of NH_2- or COOH-terminal elafin domains were incubated with *E. coli* O15 LPS at 37°C overnight in PBS containing 1mM EDTA, and mixed with SDS- and mercaptoethanol-free sample buffer to a total volume of 25μl prior to electrophoresis. Electrophoresis was performed in SDS-free 15% gels at 70V-110V for 2-3 hours, using pH 8.8 minigels as detailed in 'Materials and Methods'. Electrode buffer was SDS-free. Gels were silver stained.

inhibitor increased, an increasingly intense band was detected at the top of the gel matrices (lanes 3-7); this may represent a complex of peptide and LPS which is unable to enter the gel, perhaps due either to being too large to migrate or possessing too positive an overall charge.

Interestingly, both terminal domains of the full-length elafin molecule inhibited the migration of LPS (Figure 4), suggesting that both can bind to LPS.

It should be noted at this point that EDTA was included in reaction mixtures during these preliminary experiments, as per the technique described by Hailman *et al.* (1994) and Ding *et al.* (1999). EDTA is known to destabilise LPS (Yu and Wright, 1996), possibly by chelating divalent cations associated with the inner core region of the molecule (Rietschel *et al.*, 1994). This chelation may therefore promote elafin binding to LPS. However, later observations suggested that inclusion of EDTA was not essential for elafin-LPS interaction, and thus subsequent experiments were performed in the absence of EDTA to avoid artificial disaggregation of LPS.

It must also be emphasised that several problems arose in the execution of these experiments. For example, difficulties were encountered with the reproducibility of LPS migration, and in particular with the consistency of signal obtained with silver staining. Therefore, a number of further experiments were designed to clarify these findings.

A simple dot-blot technique was used in an attempt to demonstrate binding of full-length elafin to LPS in a simplified system. Although Figure 5 shows the results of a dot-blot investigating elafin interaction with only *E. coli* O6 LPS and *P. aeruginosa* O2 LPS, a range of LPS serotypes were used during this study (not shown). HNE was used as a positive control since elafin is known to bind to and inhibit this enzyme.

A number of difficulties were encountered in demonstrating an interaction using this method. Although it appeared at first glance that a dose response with

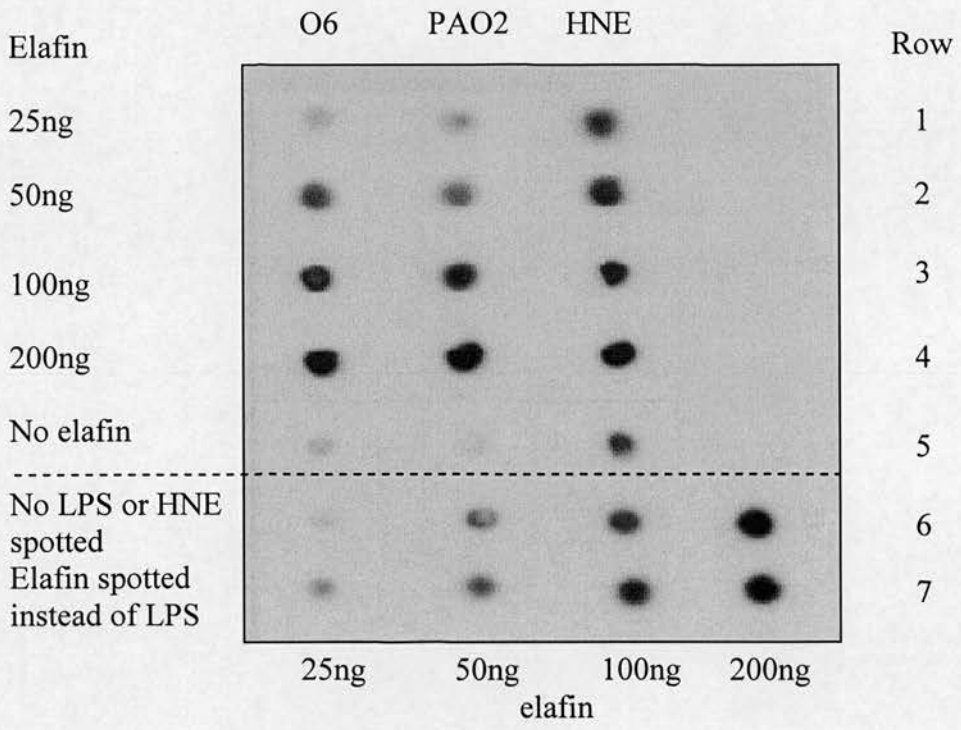


Figure 5. Non-specific binding of antibodies hinders investigation of elafin-LPS interaction by dot-blot

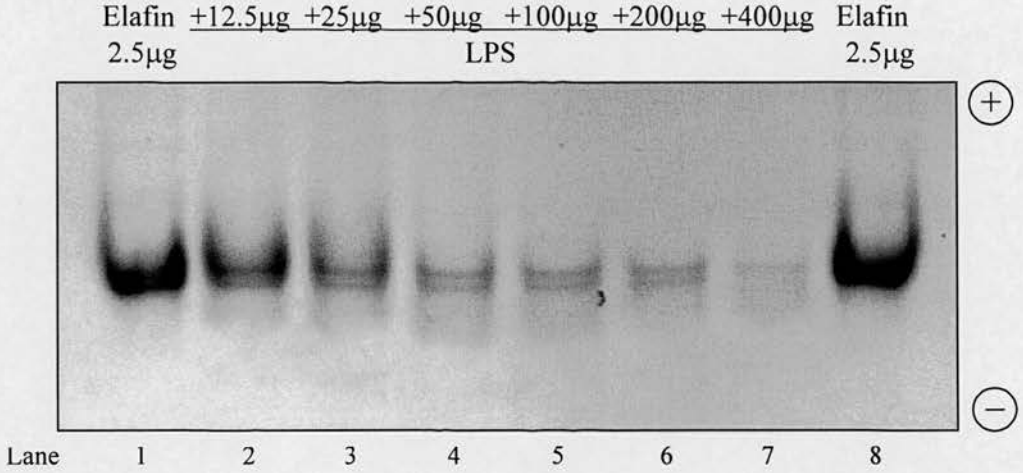
2μg of LPS or HNE were spotted onto nylon membrane and allowed to dry. Increasing amounts of elafin were dotted onto these spots, dried and the membrane blocked with PBS-Tween/5% skim milk for 1 hour at 4°C. Elafin was then detected by immunoblot analysis as described in 'Materials and Methods'. O6 = *E. coli* O6 LPS, PAO2 = *P. aeruginosa* O2 LPS. HNE was used as a positive control. Bottom two rows: row 6, no LPS or elastase spotted prior to addition of elafin; row 7, elafin spotted prior to blocking instead of LPS.

increasing elafin concentration was suggestive of binding to the immobilised LPS (rows 1-4), controls in the lower rows demonstrated problems of non-specific binding. When no elafin was spotted onto LPS or elastase, a signal was still obtained (row 5). Additionally, elafin spotted onto a blocked membrane in the absence of LPS or elastase also bound and produced a signal (row 6). It therefore appeared that dots may not necessarily be specific for elafin; indeed there appeared to be antibody cross-reactivity with both LPS and elastase (row 5), and elafin appeared to be binding directly to the blocking agent (row 6).

To assert beyond doubt that elafin and its constituent moieties interact directly with LPS, an acidic (pH 4.5) native PAGE system was utilised (see Chapter 2 for more details) (Figures 6 and 7). The pH of these gels is considerably lower than the p*I* (isoelectric point or pH) of the peptides (the p*I* of elafin is 9.7 (Wiedow *et al.*, 1990), whereas that of SLPI is 8.9 (Zitnik *et al.*, 1997)), hence they are charged positively in this buffer system and migrate to the cathode, as demonstrated by lanes 1 and 8 of each gel. LPS did not migrate in this system, as assessed by silver staining (not shown).

Full-length elafin was shown to bind to LPS; this interaction was manifested by the gradual disappearance of the characteristic elafin band in a dose-responsive manner with increasing LPS concentration (Figure 6, lanes 1-7 of upper gel). Again, SLPI acted as a positive control for this interaction and appeared to bind LPS with a higher affinity than elafin, by virtue of the loss of the SLPI band at a lower LPS concentration (Figure 6, lower gel). Both elafin NH_2- and COOH-terminal domains bound to LPS, although the affinity of the NH_2- terminal domain for LPS appeared to be much greater (Figure 7, lanes 1-7). It must be noted that, in terms of affinity of full-length elafin for LPS versus that of its constituent domains, Figures 6 and 7 cannot be compared directly. 2.5μg of full-length elafin was subjected to PAGE, contrasted with 5μg of its termini; this was due to differences in the absolute amounts of peptides required for detection by silver staining. Since each of elafin's terminal domains contribute approximately half of the entire molecule (i.e. the molecular weight of full-length elafin is 9925 Da, the NH_2-terminal domain 5172 Da,

Elafin full-length



SLPI

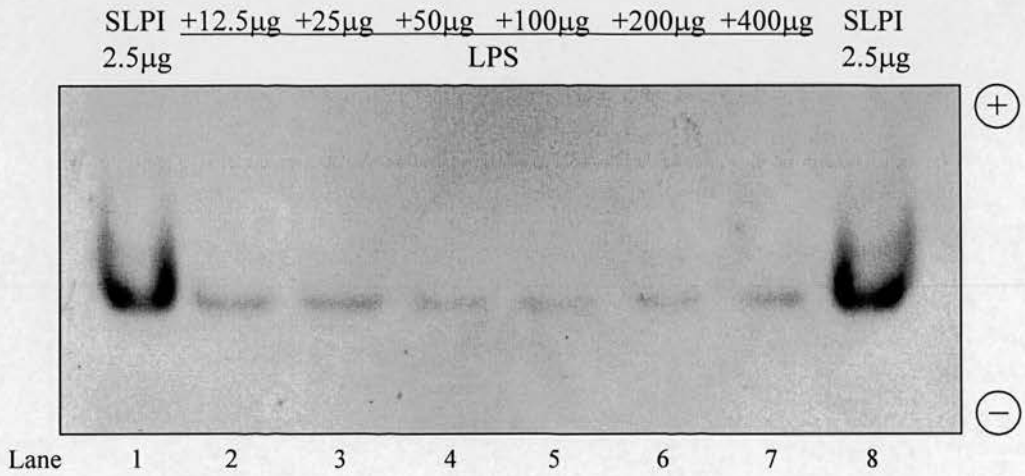
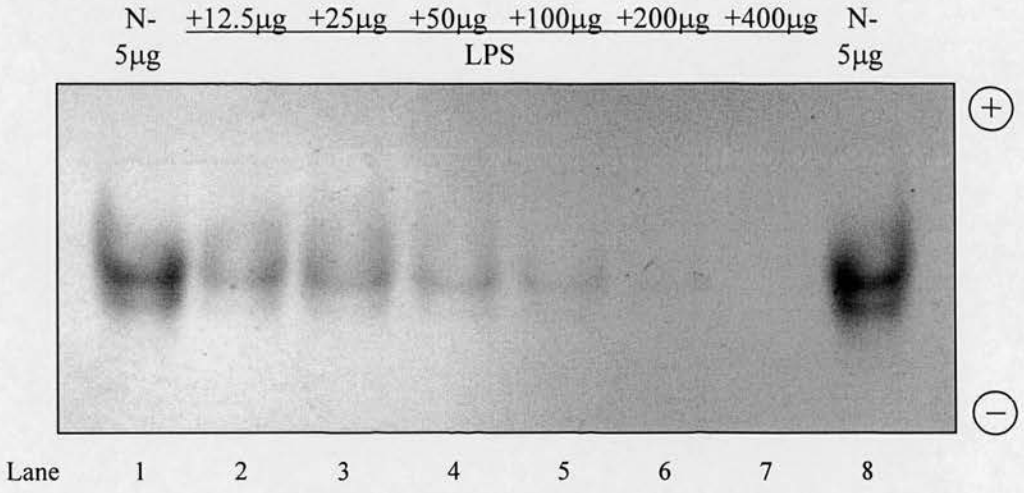


Figure 6. Direct binding of elafin to smooth-form LPS and comparison with the effects of SLPI

Native pH 4.5 PAGE of peptides followed by coomassie staining. Peptides were incubated alone or with *E. coli* O55:B5 LPS for 30 mins at 37°C in 24μl total sample volume; 6μl of sample buffer was mixed with samples prior to loading. Electrophoresis was performed in 15% gels at 80V for 2-3 hours, and peptides were detected by coomassie staining.

N-terminus of elafin



C-terminus of elafin

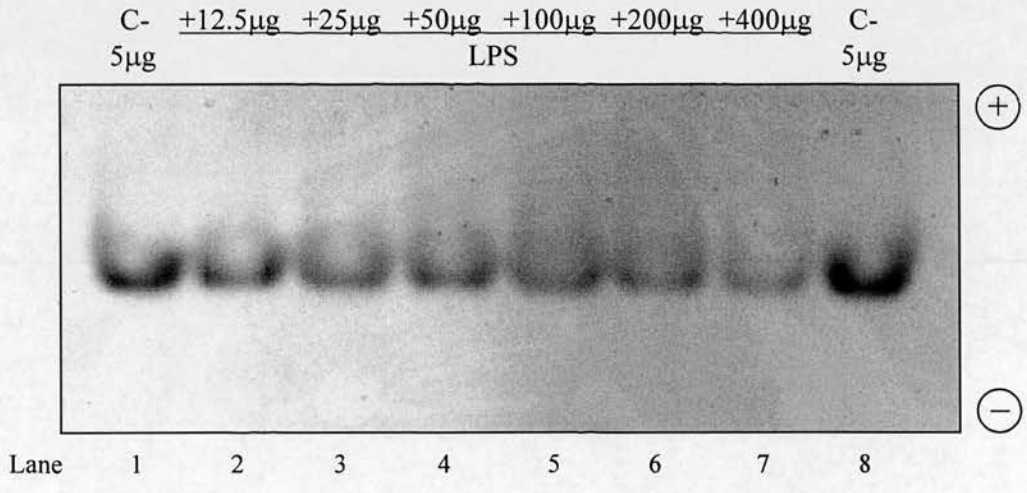


Figure 7. Direct binding of N-terminal and C-terminal elafin domains to smooth-form LPS

Native pH 4.5 PAGE of peptides followed by coomassie staining. Peptides were incubated alone or with *E. coli* O55:B5 LPS for 30 mins at 37°C in 24μl total sample volume; 6μl of sample buffer was mixed with samples prior to loading. Electrophoresis was performed in 15% gels at 80V for 2-3 hours, and peptides were detected by coomassie staining.

and the COOH-terminal domain 4776 Da), gels in Figure 7 represent around four times the molar concentration of elafin's domains as compared with the full-length molecule shown in Figure 6. This makes comparison of the whole molecule with its terminal domains difficult; however, on consideration of these acidic PAGE gels alone, it may tentatively be drawn that the affinities for LPS of the full-length molecule and its NH_2-terminal domain are similar, while that of the COOH-terminal domain is weaker than either.

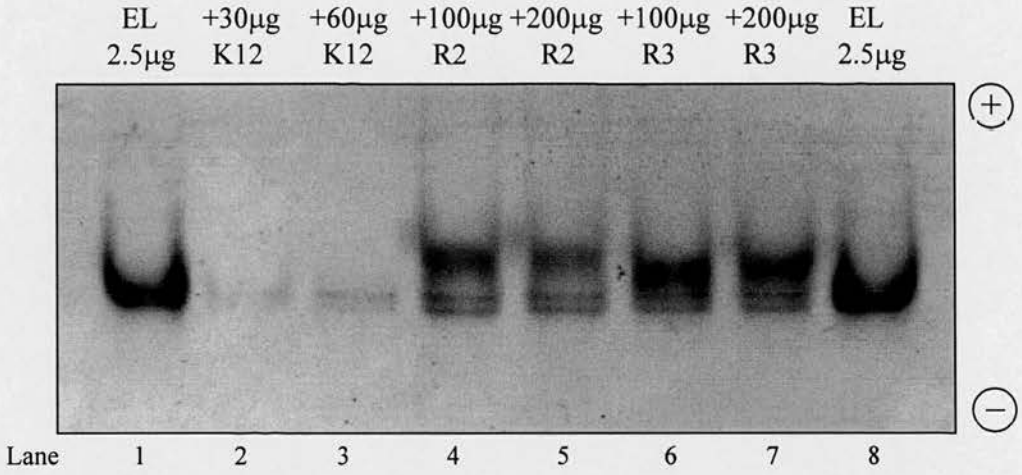
It must be noted at this point that, during the course of these investigations, difficulties were encountered in finding a suitable negative control protein for studies concerning binding to LPS (i.e. demonstration of a molecule unable to interact with LPS). These problems will be discussed briefly in the 'Discussion' section at the end of this chapter.

3.2.3. Investigation of a potential elafin binding domain within the LPS molecule

Well-characterised LPS-binding molecules such as LBP, bactericidal/ permeability-increasing protein (BPI) and polymyxin B are known to bind to LPS at a site within the conserved lipid A domain (Morrison and Jacobs, 1976; Tobias *et al.*, 1989; Gazzano-Santoro *et al.*, 1992). Initial assays to ascertain a potential elafin binding domain within the LPS molecule investigated its binding to three rough-form LPS serotypes, *E. coli* K12, R2 and R3 (discussed briefly in Chapter 1, section 1.3.2.2.); these rough-form serotypes consist solely of the lipid A-core moiety, as demonstrated in Figure 1 by the presence of only the lowest molecular weight band following PAGE (Goldman and Leive, 1987; Poxton, 1995), and therefore interaction with elafin would suggest a binding site within this region.

Elafin was found to bind strongly to K12 (Figure 8, upper gel, lanes 2 and 3). Binding to R2 and R3 was demonstrated by a dose-responsive weakening and upwards shift of the elafin band (perhaps caused by a decrease in the overall net positive charge of the elafin molecules following interaction with LPS), but did not appear to be as strong as the interaction with K12 (lanes 4-7). SLPI appeared to have

Elafin full-length



SLPI

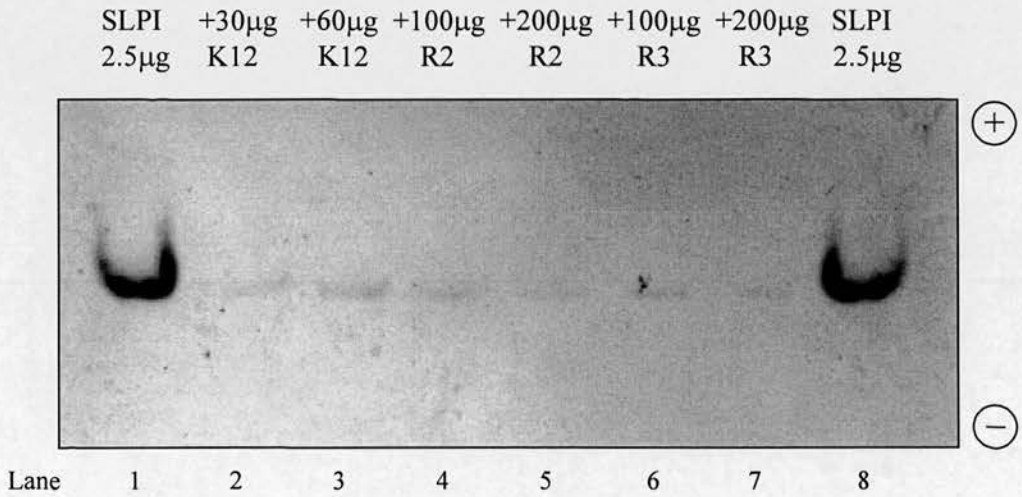


Figure 8. Binding of elafin to rough-form LPS serotypes and comparison with SLPI

Native pH 4.5 PAGE of peptides followed by coomassie staining. Peptides were incubated alone or with rough-form *E. coli* LPS serotypes K12, R2 or R3 for 30 mins at 37°C in 24μl total sample volume; 6μl of sample buffer was mixed with samples prior to loading. Electrophoresis was performed in 15% gels at 80V for 2-3 hours, and peptides were detected by coomassie staining.

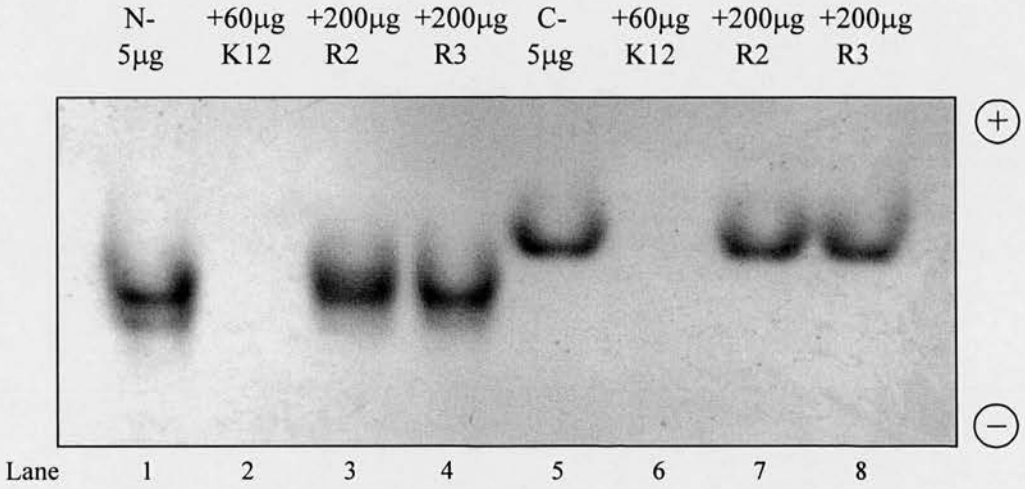


Figure 9. Binding of N-terminal and C-terminal elafin domains to *E.coli* K12 rough-form LPS

Native pH 4.5 PAGE of peptides followed by coomassie staining. Peptides were incubated alone or with rough-form *E. coli* LPS serotypes K12, R2 or R3 for 30 mins at 37°C in 24μl total sample volume; 6μl of sample buffer was mixed with samples prior to loading. Electrophoresis was performed in 15% gels at 80V for 2-3 hours, and peptides were detected by coomassie staining.

a higher affinity than elafin for rough-form LPS serotypes (Figure 8, lower gel), concurring with the findings in section 4.2.1. using the smooth-form *E. coli* serotype O55:B5.

Figure 9 demonstrates that both NH_2- and COOH-terminal elafin domains bound to K12 LPS (lanes 2 and 6), but no strong indication was observed of interaction with either R2 or R3 serotypes.

Laboratory stocks of the LPS serotypes were such that K12 could only be used in smaller quantities than R2 and R3 (Figures 8 and 9). However, the molecular weights of these rough-form serotypes are similar (approximately 2.5kDa (Srimal *et al.*, 1996; Vinogradov *et al.*, 1999)) and it was apparent that binding of elafin peptides to K12 occurred at a lower mass ratio than binding to either R2 or R3, suggestive of a greater affinity for this serotype (Figures 8 and 9).

Interaction of elafin and its terminal domains with rough-form LPS serotypes was strongly suggestive of binding to LPS via the lipid A-core portion. These findings were corroborated by further PAGE investigations using polymyxin B (a cationic cyclic polypeptide antibiotic isolated from *Bacillus polymyxa*, known to bind to lipid A with high affinity and to neutralise many of the biological effects of LPS (Morrison and Jacobs, 1976)) as a control peptide, as shown in Figures 10 and 11. LPS was separated into its 'ladder' pattern using native PAGE conditions at pH 8.8 (Figures 10A and 11A), but with SDS in electrode buffer, and silver stained. When the proteins were separated alone, these low SDS conditions hindered the migration of elafin to the anode (Figure 10A and B, lane 1) and completely inhibited migration of polymyxin B (Figure 11 A and B, lane 1).

Incubation of elafin or polymyxin B with O55:B5 or R2 LPS serotypes disrupted the LPS banding in a similar fashion, in particular by increasing the intensity of the lowest molecular weight bands near the lipid A-core region (Figures 10A and 11A), but taken alone these findings are not easily interpretable. In order to specifically detect peptide in the same gels, western blotting for elafin and

Elafin full-length

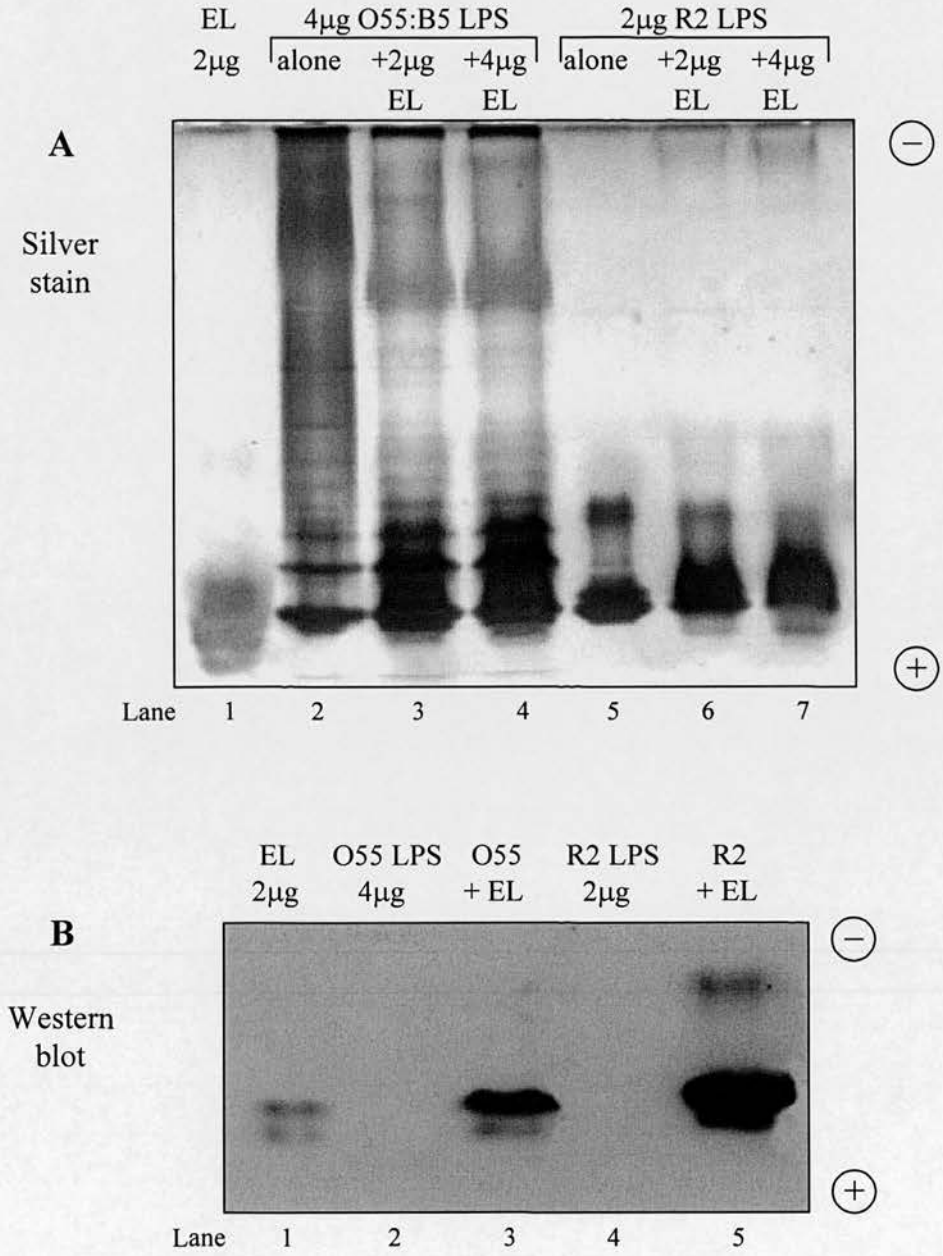


Figure 10. Elafin binds to lipid A-core portion of LPS

PAGE (pH 8.8) using SDS in electrode buffer only, followed by silver staining (A) or western blotting with anti-elafin polyclonal antibody (B). Elafin was incubated alone or with LPS for 30 mins at 37°C in 20μl total sample volume; 5μl of sample buffer was mixed with samples prior to loading. Electrophoresis was performed in 15% gels at 70V-110V for 2-3 hours.

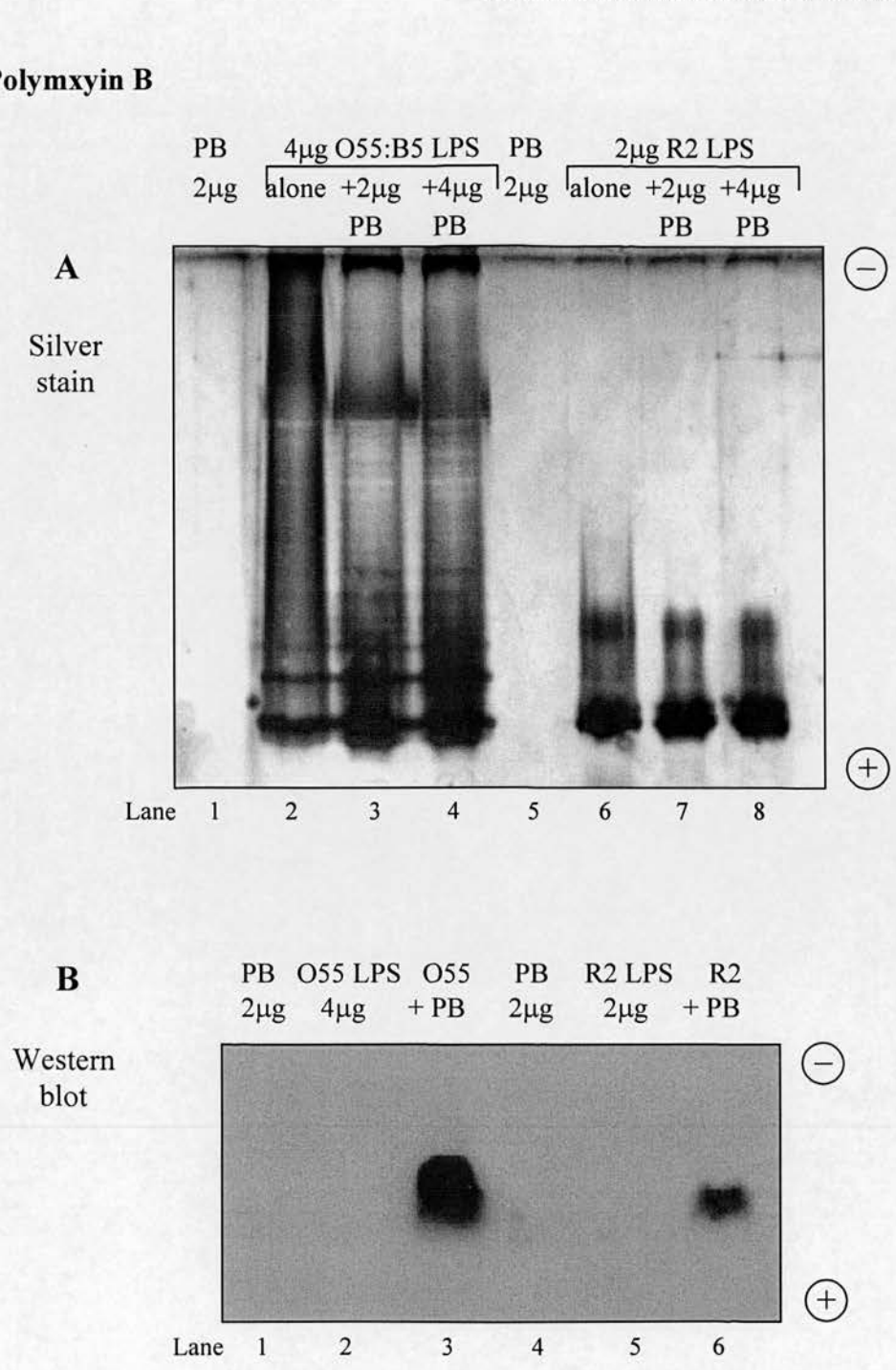


Figure 11. Binding of polymyxin B to lipid A-core portion of LPS

PAGE (pH 8.8) using SDS in electrode buffer only, followed by silver staining (A) or western blotting with anti-polymyxin B monoclonal antibody (B). Polymyxin B was incubated alone or with LPS for 30 mins at 37°C in 20μl total sample volume; 5μl of sample buffer was mixed with samples prior to loading. Electrophoresis was performed in 15% gels at 70V-110V for 2-3 hours.

polymyxin B was performed (Figures 10B and 11B). Western blotting for elafin demonstrated that the weak elafin band obtained when peptide was run alone was significantly augmented on incubation with LPS (Figure 10B); these strong bands correlated with the position of the lowest LPS band, strongly suggesting that elafin had bound to the lipid A-core moiety and migrated with LPS. Figure 11B demonstrates a similar finding in the case of polymyxin B; the peptide alone did not migrate at all, but strong bands were obtained on incubation with LPS and these again correlated with the position of the lipid A-core domain.

To extend these findings, an investigation was undertaken to study how the well-characterised LPS-binding molecules LBP and polymyxin B affected the binding of elafin to LPS; as previously mentioned, LBP and polymyxin B are known to bind LPS via the lipid A domain (Morrison and Jacobs, 1976; Tobias *et al.*, 1989).

A 1.8kDa peptide comprising an LPS-binding portion of LBP, amino acids 86-96 of the full-length molecule, was used to study the effects of LBP on elafin-LPS interaction (Figure 12). Lanes 3-5 represent molar ratios of LBP:elafin of 5:1, 10:1 and 15:1 respectively.

Binding of LBP peptide to LPS inhibited subsequent binding of elafin in a dose-responsive fashion (visualised by migration of unbound elafin), suggestive of competition for the same binding site.

Binding of both NH_2- and COOH-terminal elafin domains to LPS was also inhibited by pre-bound LBP peptide (Figure 13). Lanes 3-5 represent molar ratios of LBP:elafin peptide of 1:1, 3:1 and 5:1 respectively. Higher intensity of signal in lanes 3-5 indicates the presence of more free elafin peptides, which are prevented from binding to LPS and consequently migrate through the gel.

Additionally, studies demonstrated that polymyxin B bound to LPS could inhibit subsequent binding of either full-length elafin or its two terminal domains

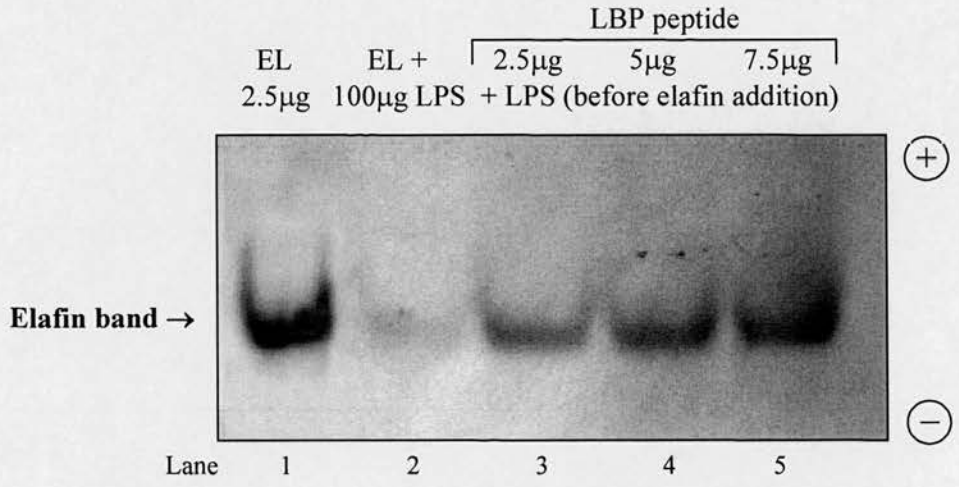
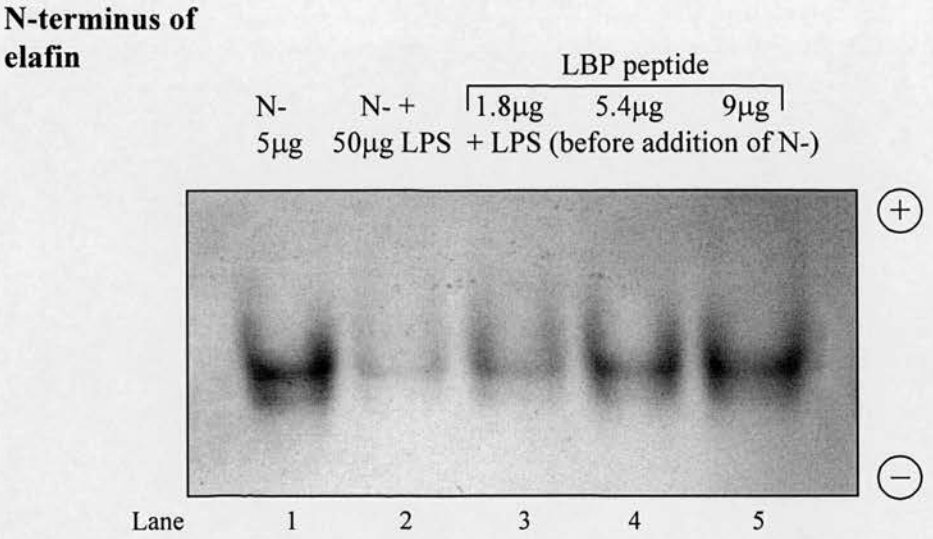


Figure 12. LBP peptide bound to LPS inhibits subsequent binding of full-length elafin

Native pH 4.5 PAGE of elafin followed by coomassie staining. Elafin was incubated alone (lane 1) or with *E. coli* O55:B5 LPS for 30 mins at 37°C (lane 2) in 24μl total sample volume; alternatively LBP peptide (aa 86-96) was incubated with LPS for 30 mins at 37°C prior to addition of elafin, followed by further incubation for 30 mins at 37°C (lanes 3-5). 6μl of sample buffer was mixed with samples prior to loading. Electrophoresis was performed in 15% gels at 80V for 2-3 hours, and elafin was detected by coomassie staining.



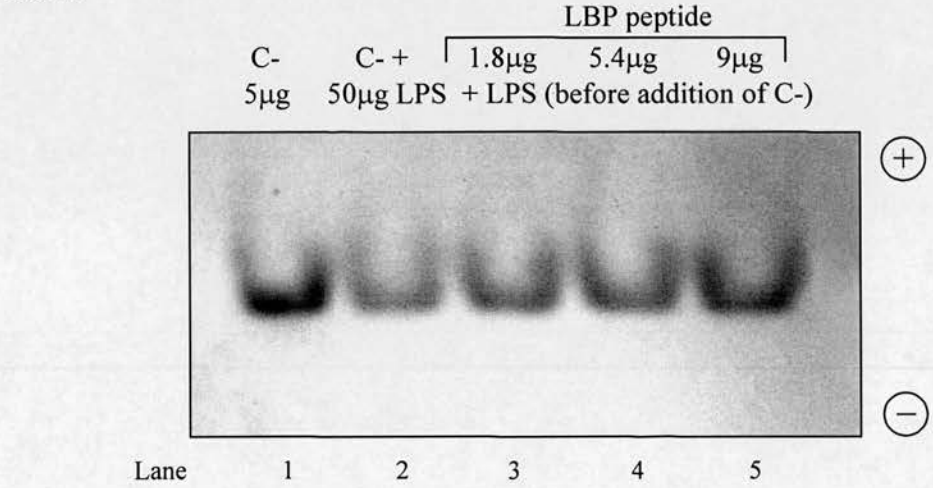


Figure 13. LBP peptide bound to LPS inhibits subsequent binding of N-terminal and C-terminal elafin domains

Native pH 4.5 PAGE of elafin peptides followed by coomassie staining. Elafin peptides were incubated alone (lane 1) or with *E. coli* O55:B5 LPS for 30 mins at 37°C (lane 2) in 24μl total sample volume; alternatively LBP peptide (aa 86-96) was incubated with LPS for 30 mins at 37°C prior to addition of elafin peptides, followed by further incubation for 30 mins at 37°C (lanes 3-5). 6μl of sample buffer was mixed with samples prior to loading. Electrophoresis was performed in 15% gels at 80V for 2-3 hours, and peptides were detected by coomassie staining.

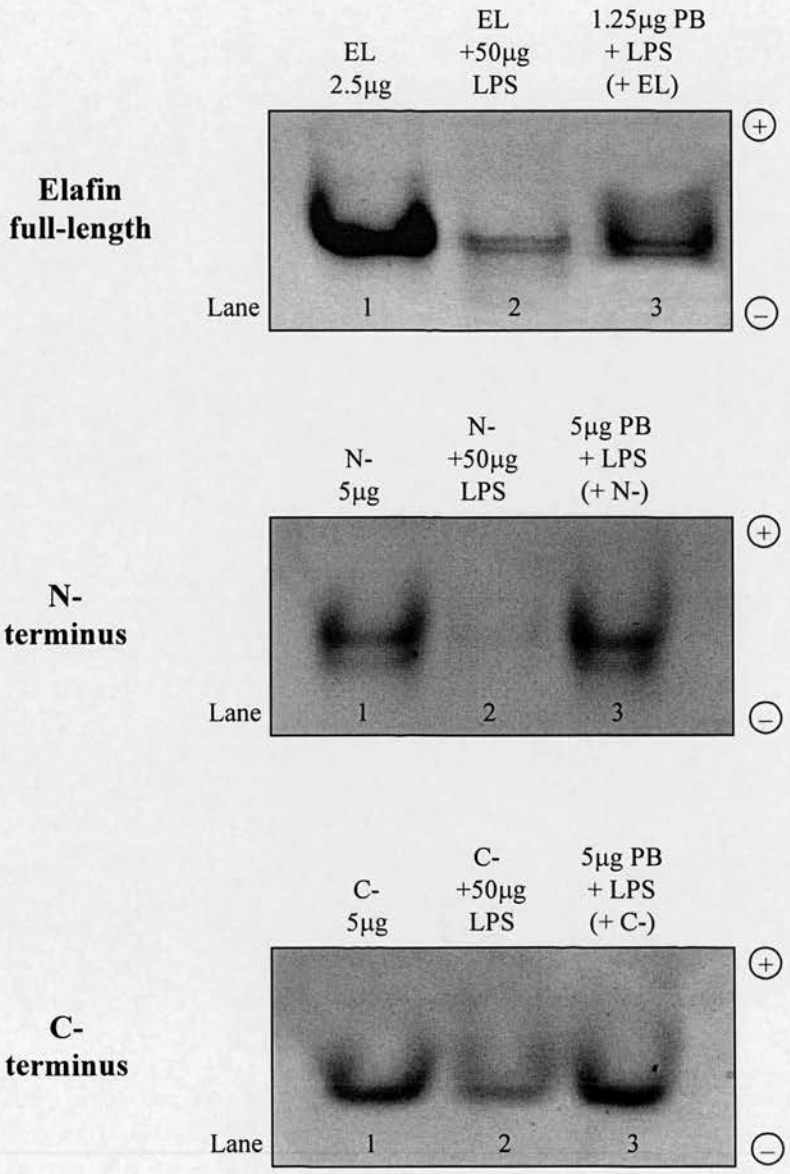


Figure 14. Polymyxin B bound to LPS inhibits subsequent binding of elafin peptides

Native pH 4.5 PAGE of elafin peptides followed by coomassie staining. Elafin peptides were incubated alone (lane 1) or with *E. coli* O55:B5 LPS for 30 mins at 37°C (lane 2) in 24µl total sample volume; alternatively polymyxin B was incubated with LPS for 30 mins at 37°C prior to addition of elafin peptides, followed by further incubation for 30 mins at 37°C (lane 3). 6µl of sample buffer was mixed with samples prior to loading. Electrophoresis was performed in 15% gels at 80V for 2-3 hours, and peptides were detected by coomassie staining.

(Figure 14). Polymyxin B was bound to LPS at a molar ratio of 5:1 to elafin or its terminal domains.

These findings indicate that elafin, LBP and polymyxin B may compete for a similar binding site within the LPS molecule.

3.2.4. Effects of elafin on the interaction of LPS with LBP

Having established that elafin binds directly to LPS and may compete with LBP for a binding site within the LPS molecule, experiments were designed to investigate whether elafin could preclude the binding of LPS to LBP. An ELISA technique measuring the binding of biotinylated LPS to immobilised LBP was used for these studies; this technique has previously been used to demonstrate that several cationic antimicrobial peptides can inhibit the LPS-LBP interaction (Scott *et al.*, 2000b).

Binding of LPS to concentrations of LBP ranging from 0-50ng/ml produced a linear standard curve (Figure 15). For the purpose of investigation of the effects of elafin on this interaction, 25ng/ml LBP was used throughout.

Initial experiments investigated the effects of elafin peptide concentrations ranging from 0.625-10μM (Figure 16). SLPI was also used since, although it has previously been shown by others to bind to LPS, its effects on the binding of LPS to LBP have not been elucidated. Over these high concentrations, both the NH_2- and COOH-terminal elafin domains markedly inhibited the LPS-LBP interaction, though the COOH-terminal domain was the more potent inhibitor. Inhibition by both NH_2- and COOH-terminal domains was maximal at 5μM, eliciting 51% and 73% inhibition respectively. Interestingly, inhibition in both cases decreased at 10μM. Inhibition of the LPS-LBP interaction by SLPI was dose-responsive to a maximal 76% inhibition at 10μM. Polymyxin B, incorporated as a positive control for inhibition of LPS-LBP interaction, was the most potent inhibitor over these concentrations to a maximal 89% at 10μM. Intriguingly, Figure 16 demonstrates that

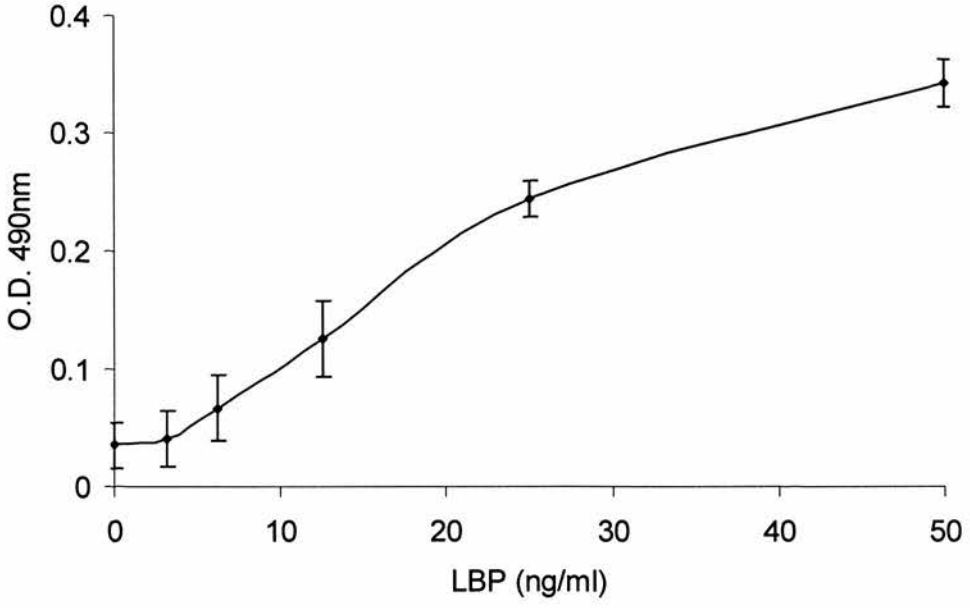


Figure 15. Standard curve of binding of LBP to LPS

Biotinylated *E. coli* O55:B5 LPS was added to wells containing increasing concentrations of recombinant LBP bound to anti-LBP monoclonal antibody. Binding of LPS to LBP was detected using HRP-conjugated streptavidin and absorbance read at 490nm. Values represent mean ± S.E. of n=3 experiments, each performed in duplicate.

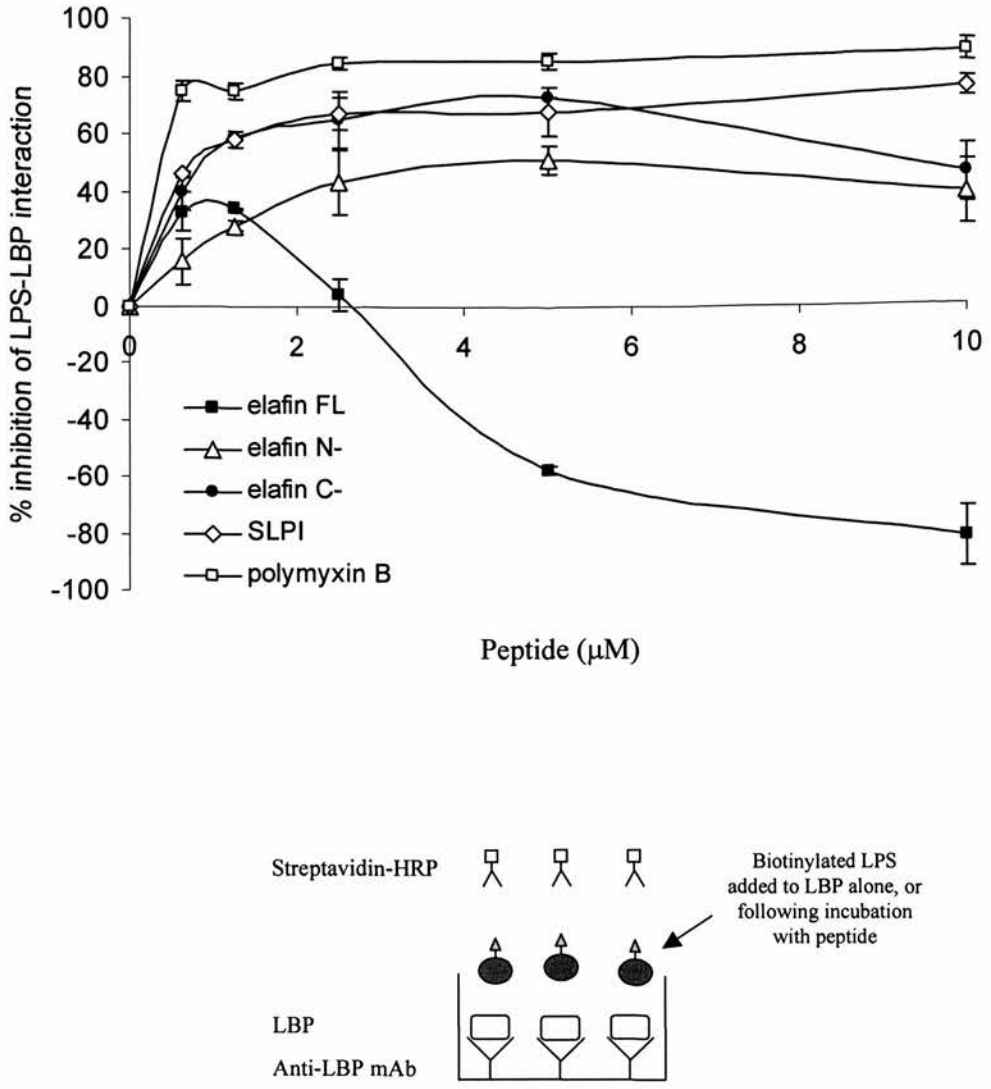


Figure 16. Effects of 0.625-10μM elafin peptides on the binding of LPS to LBP

Biotinylated *E. coli* O55:B5 LPS or biotinylated LPS pre-incubated with peptide for 30mins at 37°C was added to wells containing 25ng/ml recombinant LBP, immobilised using anti-LBP monoclonal antibody. Binding of LPS to LBP was detected using HRP-conjugated streptavidin and absorbance read at 490nm. ELISA technique is depicted diagrammatically beneath graph. For a more detailed description of this technique see Chapter 2, section 2.2.1.7. Values represent mean ± S.E. of n=3 experiments, each performed in duplicate. Where no error bars are shown, they were smaller than the symbols.

full-length elafin inhibited the LPS-LBP interaction only at the lowest concentrations, and in fact markedly enhanced the level of LPS detectable in wells by up to 82% at 10μM elafin. This finding will be discussed in the 'Discussion' section at the end of this chapter.

On the basis of these findings, and considering that all peptides inhibited the LPS-LBP interaction to some extent at lower concentrations as demonstrated by Figure 16, the concentration of test peptides was reduced to the range 9.75nM-156nM (Figure 17). This concentration range also relates more closely to the ratios of elafin:LPS investigated previously using PAGE techniques (Figures 3, 4 , 6 and 7). For instance, in this ELISA system, a constant 270nM LPS was added to test wells following incubation with the varying concentrations of full-length elafin, such that the molar ratios of full-length elafin:LPS in Figure 17 range from 1:28 to 1:1.7. Conversion of the fixed concentration of LPS separated by basic (pH 8.8) PAGE in Figures 3 and 4 to a molarity (20μg/20μl sample volume ≈ 48.8μM), and conversion of the range of full-length elafin concentrations incubated with LPS (250ng to 5μg/20μl sample volume ≈ 1.25μM to 25μM), indicates that the molar ratios of elafin:LPS tested by basic PAGE ranged from 1:39 to 1:2. Similarly, conversion of the constant full-length elafin concentration resolved by acidic PAGE in Figure 6 to a molarity (2.5μg/24μl sample volume ≈ 10.4μM), and conversion of the range of LPS concentrations incubated with elafin (12.5μg to 400μg/24μl sample volume ≈ 25.4μM to 813μM), demonstrates that the molar ratios of elafin:LPS investigated by acidic PAGE ranged from 1:78 to 1:2.4. Thus, the molar ratios of elafin:LPS tested by PAGE (Figures 3, 4, 6 and 7) and ELISA (Figure 17) are largely comparable.

In the concentration range 9.75nM-156nM, all forms of elafin inhibited the LPS-LBP interaction (Figure 17). The patterns of inhibition of NH_2- and COOH-terminal domains were very similar across the concentration range, although the COOH-terminal domain was the more effective inhibitor below 39nM; maximal inhibition by both termini was around 47% at 78nM. At low concentrations, inhibition effected by full-length elafin was comparable with that of the NH_2-terminal domain but was weaker than either of its constituent termini at intermediate

concentrations; maximal inhibition by full-length elafin was obtained at 156nM. Inhibition of LPS-LBP binding by all three elafin peptides was more or less identical at 156nM (around 45%).

SLPI also inhibited the LPS-LBP interaction maximally at 156nM. The dose-response curves for SLPI and full-length elafin were similar, however elafin's NH_2- and COOH-terminal domains appeared to be more powerful inhibitors than SLPI over these concentrations.

As was also the case at higher concentrations shown in Figure 16, polymyxin B was the most powerful inhibitor overall. Greatest inhibition of LPS-LBP binding by polymyxin B was 62% at 156nM.

Separate experiments were designed to investigate the relative affinities of elafin and LBP for binding to LPS. Using an ELISA technique measuring elafin bound to immobilised LPS, LBP was shown to have a stronger affinity for LPS than that of elafin for LPS (Figure 18). Elafin pre-bound to LPS was displaced on addition of LBP, while simultaneous addition of elafin and LBP to LPS resulted in a 90% reduction in elafin-LPS binding (as compared with binding in the absence of LBP).

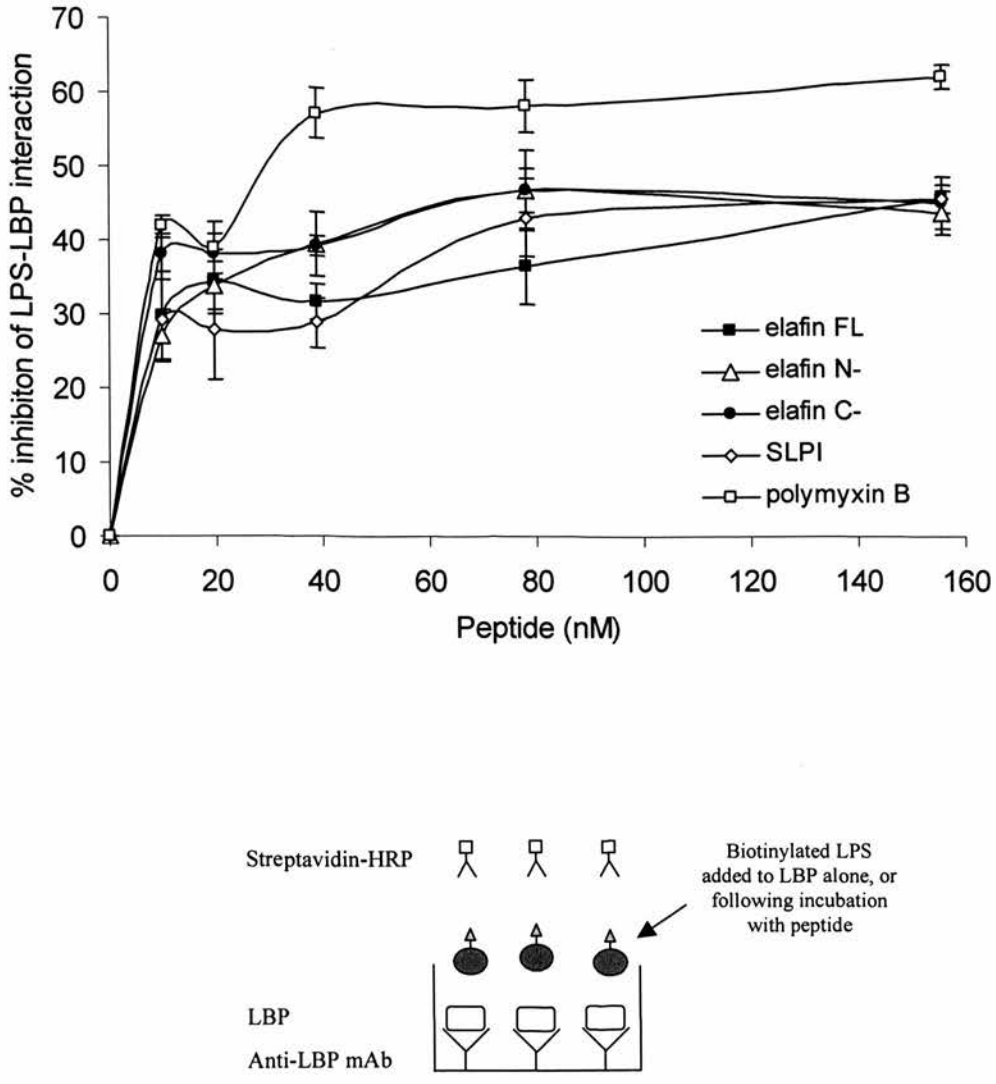


Figure 17. Elafin peptides in the concentration range 9.75-156nM inhibit the binding of LPS to LBP

Biotinylated *E. coli* O55:B5 LPS or biotinylated LPS pre-incubated with peptide for 30mins at 37°C was added to wells containing 25ng/ml recombinant LBP, immobilised using anti-LBP monoclonal antibody. Binding of LPS to LBP was detected using HRP-conjugated streptavidin and absorbance read at 490nm. ELISA technique is depicted diagrammatically beneath graph. For a more detailed description of this technique see Chapter 2, section 2.2.1.7. Values represent mean ± S.E. of n=3 experiments, each performed in duplicate.

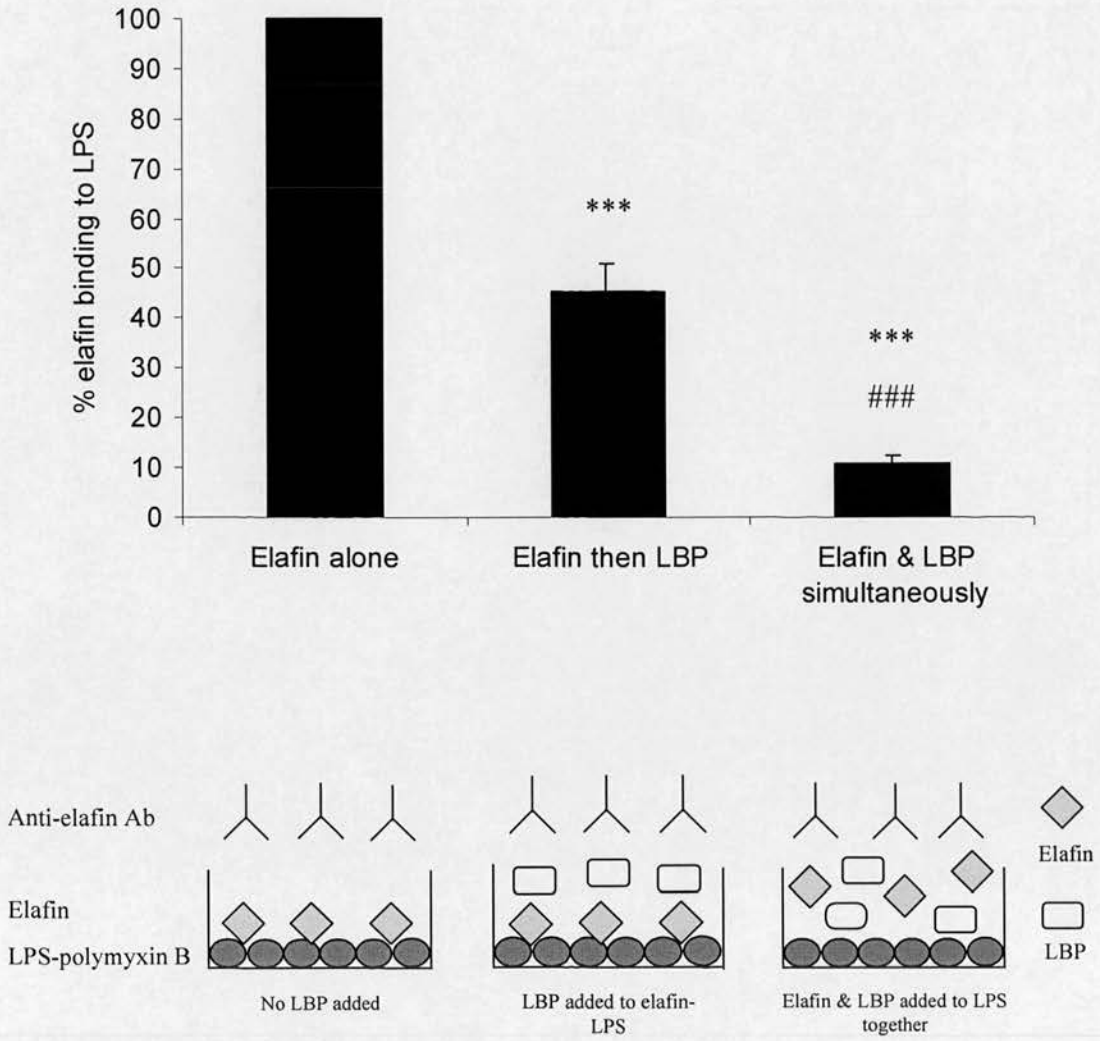


Figure 18. Elafin binding to LPS is out-competed by LBP

Elafin (156nM) was added to wells containing *E. coli* O55:B5 LPS immobilised using polymyxin B. Alternatively, 25ng/ml LBP was added to wells containing elafin pre-incubated with LPS, or elafin and LBP were added to wells simultaneously. Binding of elafin to LPS was detected using anti-elafin polyclonal antibody followed by an HRP-conjugated secondary antibody; absorbance was read at 490nm. Major ELISA components are depicted diagrammatically beneath graph. For a more detailed description of this technique see Chapter 2, section 2.2.1.8. Values represent mean ± S.E. of n=4 experiments, each performed in duplicate. *** = significant difference, $P<0.001$, compared with 'elafin alone'. ### = significant difference, $P<0.001$, compared with 'elafin then LBP'.

3.3. DISCUSSION

The principal novel observation arising from the work in this chapter is that elafin is capable of direct interaction with Gram-negative bacterial LPS. Furthermore, this binding appears to take place within the conserved lipid A domain of the LPS molecule. As a direct consequence of its interaction with LPS, elafin is capable of inhibiting subsequent binding of LPS to the lipid transfer protein LBP, a central step in LPS-mediated cellular activation (Tobias and Ulevitch, 1986; Schumann *et al.*, 1990).

It seems important to point out at this point that this study did not set out to investigate the kinetics of elafin's interactions with LPS. The foremost objective of the work in this chapter was to determine whether or not elafin could directly bind to LPS, and secondarily to identify to which region of the LPS molecule elafin may bind. By answering these initial questions, it was hoped that we could go on to reveal potential roles for elafin in modulating LPS-mediated inflammation. The author is aware that the use of techniques such as saturation radioligand binding assays would perhaps allow us to characterise the interactions of elafin with LPS more specifically, but for the purposes of our investigation such studies were considered to be unnecessary.

Before going on to consider the findings of this chapter in more depth, the following section provides a brief discussion concerning the use of peptide controls for these studies.

3.3.1. Technical considerations – control proteins

As mentioned within the results section of this chapter, difficulties were encountered in finding a suitable negative control for these studies. Polymyxin B and SLPI were used as positive controls throughout the course of this investigation, since these molecules had previously been shown to possess LPS-binding properties. However, identifying a 'passive' protein which did not bind to LPS proved difficult.

The 14kDa cationic protein cytochrome c (an essential component of the mitochondrial respiratory chain; for a brief review see Green *et al.*, 1998) was chosen due to its cationicity and similar molecular weight to elafin. However, cytochrome c bound to LPS directly and could also inhibit the interaction of LPS with LBP (data not shown). Similarly, the cationic antimicrobial peptide protamine (Johansen *et al.*, 1997) was found to bind to LPS (not shown). Interestingly, since the completion of the studies described herein, a study has been documented describing the LPS-binding activity of protamine and its pro-inflammatory effects in LPS-activated whole blood (Bosshart *et al.*, 2002).

Finally, bovine serum albumin (BSA) was shown to be capable of binding to LPS, and could effectively inhibit the LPS-LBP interaction (data not shown). Recent work has demonstrated that human serum albumin can bind to rough-form LPS and lipid A (Jurgens *et al.*, 2002). Moreover, another study published since cessation of this investigation has implicated a role for albumin in interactions of endotoxin with LBP and soluble CD14 (Gioannini *et al.*, 2002), suggesting that albumin may not be an innocent bystander in LPS-mediated cellular activation.

Thus, it appears that a plethora of molecules can bind to LPS and modulate its effects to some degree. It is interesting to note that the majority of published works investigating the effects of cationic antimicrobial peptides on LPS activity do not include a negative control, and it may therefore be the case that others have encountered similar difficulties. For the purpose of these studies, we have considered the terminal domains of elafin as 'internal' controls for the binding of the full-length molecule to LPS, and in this regard several observations have provided evidence for specificity in LPS-binding activity. The findings of this investigation are discussed in the following section.

3.3.2. Interactions of elafin with LPS

Prior to commencing investigation of the LPS-binding properties of elafin, it was deemed necessary to gain an understanding of the characteristics of LPS within the PAGE conditions used for subsequent studies. Figures 1 and 2A demonstrate the

inherent complexity and variation of LPS structure amongst serotypes, particularly with regard to composition of the diverse O-specific polysaccharide side-chain and consequent molecular weight. The classical 'ladder' pattern observed in these figures can be attributed to assembly of a range of O-polysaccharide lengths by a particular bacterial strain due to incomplete chain synthesis (Erridge *et al.*, 2002). As discussed in detail in Chapter 1, the general LPS structure consists of three distinct regions: (i) the highly hydrophobic lipid A domain responsible for the endotoxic properties of the molecule, (ii) the core oligosaccharide consisting of inner and outer core portions, and (iii) the serotype-specific O-polysaccharide side-chain (Rietschel *et al.*, 1992). Appendix I at the end of this chapter provides a schematic diagram of this general LPS structure, as shown previously in Chapter 1. The heterogeneity of LPS within the Enterobacteriaceae family is demonstrated by PAGE in Figure 1. *E.* coli K12, R2 and R3 are rough-form LPS serotypes and as such contain only lipid A-core oligosaccharide (represented by the lowest band in each lane), whereas the smooth-form *E.* coli serotype O55:B5 also consists of a number of O-polysaccharide repeats; each subsequently larger band in the O55:B5 lanes corresponds to lipid A-core oligosaccharide bound to an extra O-antigen unit (Goldman and Leive, 1987).

Well-characterised LPS-binding molecules such as LBP, BPI and polymyxin B are known to bind to the conserved lipid A portion of LPS (Morrison and Jacobs, 1976; Tobias *et al.*, 1989; Gazzano-Santoro *et al.*, 1992), however the precise binding sites of these molecules within the lipid A structure are unclear. For example, a study carried out by Morrison and Jacobs (1976) concluded that polymyxin B binds to lipid A-Kdo, as they demonstrated interaction with LPS derived from *Salmonella minnesota* Re595, a mutant strain unable to synthesise heptose. However, most recent studies concur that the minimal LPS structure to which LBP, BPI and polymyxin B bind is the acylated glucosamine disaccharide unit of lipid A (Gazzano-Santoro *et al.*, 1992; Theofan *et al.*, 1994; Srimal *et al.*, 1996; Kellogg *et al.*, 1999; Kellogg *et al.*, 2001). Furthermore, it has been demonstrated that BPI and LBP compete for binding to identical or overlapping determinants within the LPS molecule; these studies also suggested that the affinity of BPI for LPS is 20-100-fold greater than that of LBP (Gazzano-Santoro *et al.*, 1994; Wilde *et*

al., 1994). The binding site for polymyxin B also appears to overlap with those of LBP and BPI, since binding of BPI to LPS is inhibited by polymyxin B (Schafer *et al.*, 1984).

The ability to bind to the conserved lipid A portion of LPS is therefore considered a requisite of anti-endotoxin agents, and it is this characteristic which enables LPS-binding molecules to interact with a broad range of heterogeneous serotypes.

However, in advance of exploring the possibility of elafin interaction with smaller structural determinants of the LPS holo-molecule, binding to smooth-form serotypes was investigated. *E. coli* LPS was chosen for these studies for a number of reasons. Firstly, as a major gut commensal, *E. coli* LPS is recognised as a prominent causative agent of septic shock following translocation from the gastrointestinal tract (Bochud *et al.*, 2001; Erridge *et al*, 2002). Secondly, the lipid A of *E. coli* LPS is considered to represent the optimal structure for induction of the immune response i.e. endotoxicity; this structure consists of hexa-acylated (chain length C12-C14) lipid A containing a bis-phosphorylated β(1-6)-linked D-glucosamine disaccharide unit (Rietschel *et al.*, 1993; Rietschel *et al.*, 1994). Thirdly, LPS of *E. coli* has been extensively studied in terms of both structure and activity, and considerable expertise in the field of pathogenicity of the Enterobacteriaceae exists locally within Edinburgh University (Poxton , 1995; Currie *et al.*, 2001).

Full-length elafin bound directly to smooth-form LPS and this interaction was demonstrated using two different PAGE-based techniques (Figures 3 and 6). These native PAGE assays were utilised to minimise inclusion of SDS in the systems, since as an anionically-charged detergent SDS was deemed a potential impediment to interaction of cationic peptides and anionic LPS molecules. The interaction of elafin with LPS was detected either by inhibition of LPS migration in pH 8.8 native PAGE gels (Figure 3), or by inhibition of elafin migration in pH 4.5 native PAGE gels (Figure 6), and in both systems the interaction was dose-dependent. Interaction of SLPI with LPS was also investigated (Figures 3 and 6), and in this regard SLPI was

used as a positive control since direct binding of SLPI to LPS has previously been demonstrated by Ding *et al.* (1999); Ding *et al.* used a silver-stained PAGE technique similar to that employed in Figure 3, and this study corroborates their findings.

Although Figure 3 initially suggested that elafin's interaction with LPS is of a greater affinity than that of SLPI with LPS, Figure 6 strongly suggests that SLPI interacts with LPS more strongly than elafin; the intense SLPI band observed in the absence of LPS (lanes 1 and 8) was inhibited at a much lower dose of LPS than was the case with elafin. Although several factors could account for this disparity of affinity for LPS, net charge of the proteinase inhibitors may play a role in these findings. The overall net charge of the full-length elafin molecule is +7, whilst that of SLPI is +12 (these charges were determined using the amino acid sequences given by Sallenave and Silva (1993) and Thompson and Ohlsson (1986) respectively). However, if indeed charge were the sole determinant of affinity for binding to anionic targets in the LPS structure, SLPI's affinity for LPS would be expected to be almost twice that of elafin. Figure 6 suggests that this is not the case, since at least 200μg of LPS was required to elicit a similar inhibition of elafin migration to that obtained with 12.5μg LPS in the case of SLPI; this observation implies that SLPI affinity for LPS is more than double that of elafin. Thus, although the cationicity of elafin and SLPI may be an important determinant of binding to LPS, other properties of the molecules may be required for interaction.

Interestingly, both NH_2- and COOH-terminal domains of elafin were demonstrated to bind to smooth-form LPS (Figures 4 and 7). The NH_2-terminal domain was found to bind to LPS more strongly than the COOH-terminal domain. Previous work has shown that the anti-proteinase activity of elafin resides exclusively within the COOH-terminal domain, whilst both domains harbour inherent anti-microbial activity (Simpson *et al.*, 1999). The study by Simpson *et al.* (1999) demonstrated that the majority of elafin's antimicrobial activity against Gram-negative *P. aeruginosa* is located within the NH_2-terminal domain; this portion of elafin has a net charge of +5, while that of the COOH-terminus is +2. Although charge was shown not to be the sole determinant of antimicrobial activity in the

Simpson *et al.* study (indeed, COOH-terminal elafin displayed more potent activity than the NH_2-terminus against the Gram-positive pathogen *S. aureus*), our initial hypothesis prior to this study was that the NH_2-terminal domain would be the major site of LPS-binding, due to its greater cationicity and antimicrobial activity against Gram-negative organisms. Whilst it appears that the NH_2-terminal domain does indeed harbour the majority of elafin's LPS-binding activity, the COOH-terminus also appears to play a role in the activity of the full-length molecule.

These findings were further extended by investigation of binding of elafin to smaller fragments of the LPS molecule. Full-length elafin interacted directly with rough-form K12, R2 and R3 *E. coli* LPS serotypes lacking the O-specific side-chain (Figure 8). These serotypes represent three of the five core oligosaccharide types in *E. coli* (the other two being R1 and R4) and are constructed of lipid A bound to core oligosaccharide (Amor *et al.*, 2000). These regions are far more conserved than the hypervariable O-polysaccharide antigens, of which there are more than 170 in *E. coli*, and this lack of diversity in lipid A-core oligosaccharide can be attributed to the constraints imposed by their essential role in outer membrane stability (Heinrichs *et al.*, 1998). Binding of elafin to these core types strongly suggests that interaction of elafin with smooth-form LPS results from interaction with components within the lipid A-core region, structurally conserved between species and serotypes. Interestingly, elafin appeared to bind to K12 more strongly than either R2 or R3. This finding can perhaps be explained by analysis of the outer core structure of the core types (as detailed by Nnalue *et al.* (1999), Vinogradov *et al.* (1999) and Amor *et al.* (2000)). Appendix II following this chapter provides the structures of the K12, R2 and R3 outer core oligosaccharides, in addition to the chemical structure of *E. coli* lipid A (these structures were previously shown in Chapter 1, along with the structures of R1 and R4). All three of these outer core oligosaccharides consist of three hexose sugars attached to two side chain residues. In the cases of R2 and K12, the hexose sugars are all glucose residues, while the second glucose is replaced by galactose in R3. The side-chain residues of the core types are as follows: galactose and *N*-acetyl glucosamine in R2; *N*-acetyl glucosamine and glucose in R3; and galactose and heptose in K12. K12 therefore contains a fourth heptose residue (in

addition to the triplet of heptoses found in the inner core of all 5 *E. coli* core oligosaccharide types), and this heptose is partially substituted by a β-linked *N*-acetyl glucosamine. It may potentially be this side-chain which results in higher affinity binding of elafin to K12 core oligosaccharide, by providing an additional sugar residue for elafin interaction; this residue may also be substituted with charged groups, such as phosphate, with which elafin may interact electrostatically (Erridge *et al.*, 2002).

Although these findings already strongly indicated that elafin binds directly to a site within the lipid A-core portion, PAGE techniques designed to separate LPS into its 'ladder' pattern confirmed, using polymyxin B as a positive control, that elafin indeed binds to and migrates with the lowest molecular weight moiety of both smooth-form and rough-form serotypes (Figures 10 and 11).

Both the NH_2- and COOH-terminal elafin domains bound to K12 LPS, but interaction with either R2 or R3 could not be demonstrated (Figure 9). On comparison of Figures 8 and 9, it must be noted that the molar concentration of terminal domains used was four times that of full-length elafin; thus the ratio of peptide:LPS was greater in Figure 9, suggesting perhaps the dose of R2 and R3 serotypes may not have been sufficient to inhibit peptide migration. In any case, the previous observations concerning structural diversity of the core oligosaccharide types may also account for the greater affinity of elafin's terminal domains for K12.

SLPI also bound rough-form LPS and, as was found to be the case with smooth-form serotypes, this interaction was of a greater higher affinity than that of elafin and LPS (Figure 8). These findings suggest that elafin and SLPI bind LPS within a similar portion of the LPS molecule.

The discovery that elafin, and its constituent terminal domains, could be precluded from binding to LPS by peptides known to bind LPS within the lipid A domain provided strong evidence that elafin binds directly to the highly conserved LPS backbone of acylated glucosamine disaccharide (Figures 12-14); this suggests

that, although affinity for different core types may vary due to the nature of hexose residues and side-chains, binding to LPS occurs regardless of core oligosaccharide.

The LBP peptide portion used to inhibit binding of elafin peptides to LPS in Figures 12 and 13 constitutes amino acids 86-99 of the molecule, and binds directly to lipid A. This portion of the LBP molecule is highly cationic (net charge +6) due to the presence of three arginine residues and three lysine residues, and the basic residues in this region have been demonstrated previously to play an important role in the interactions of LBP with LPS (Lamping *et al.*, 1996). The NH_2-terminal domain (amino acids 1-197) is known to possess the LPS-binding properties of the holo-molecule, while the COOH-terminal domain (amino acids 198-456) is important for transfer of LPS to CD14 (Han *et al.*, 1994; Thoefan *et al.*, 1994; Taylor *et al.*, 1995; Abrahamson *et al.*, 1997). The abundance of glutamate and aspartate residues throughout the molecule reduces its overall charge to +4, and this net positive charge resides entirely within the NH_2-terminal domain since the charge on the COOH-terminal domain is neutral (determined by analysis of the complete amino acid sequence of LBP detailed by Wilde *et al.* (1994)).

While it is widely accepted that the binding domain for LBP within the LPS molecule lies within lipid A, the structural features of lipid A which are important for binding by LBP have not been well studied. However, a study by Gazzano-Santoro *et al.* (1995) characterised the lipid A elements which are important for binding of $rBPI_{23}$, a recombinant fragment of the homologous protein BPI, corresponding to the NH_2-terminal domain of the molecule (amino acids 199-456). BPI is a potently Gram-negative bactericidal protein stored in azurophilic granules of polymorphonuclear leukocytes which, in contrast to LBP, neutralises the biological activities of LPS (Elsbach, 1998). The study by Gazzano-Santoro *et al.* (1995) found that binding of $rBPI_{23}$ to lipid A was dependent upon two negatively charged moieties, usually but not necessarily phosphate, attached to the 1 and 4′ hydroxyl groups of the glucosamine disaccharide unit, and also upon the presence of fatty acid chains attached to the disaccharide (Gazzano-Santoro *et al.*, 1995). Since LBP and BPI share approximately 45% sequence homology at the amino acid level, and the

predominant divergences within their sequences are located in the COOH-terminal domains (Gray *et al.*, 1989; Beamer *et al.*, 1998), it is perhaps reasonable to surmise that the same structural determinants of lipid A may be important for binding of LBP.

The inhibition of binding of full-length elafin (Figure 12) and both NH_2- and COOH-terminal elafin domains (Figure 13) to LPS pre-bound to LBP peptide suggests that these structural determinants within lipid A (described above) may also play a part in the interaction of elafin with lipid A. Indeed, the observation that the cyclic cationic decapaptide antibiotic polymyxin B also prevents interaction of elafin peptides with LPS adds further support to this theory (Figure 14). The binding of polymyxin B to lipid A has been extensively investigated, and both electrostatic interactions with phosphoryl groups and hydrophobic interactions involving acyl chains of lipid A have been shown to be important (Rustici *et al.*, 1993; Srimal *et al.*, 1996; Thomas *et al.*, 1998; Yin *et al.*, 2003).

It therefore appears that elafin binds LPS within a portion of lipid A commonly targeted by LPS agonists and antagonists alike. It seemed logical to extend these findings to an investigation of the effects of elafin on the interaction of LPS with LBP, since this step is central to the initiation of cellular responses to LPS (as discussed in Chapter 1). A recent study by Scott *et al.* (2000b) described the ability of a number of structurally diverse cationic antimicrobial peptides, including HBD-2, HNP-1 and polymyxin B, to block the binding of LPS to LBP. A similar assay was used here to reveal that full-length elafin and both its NH_2- and COOH-terminal domains can also inhibit the interaction of LPS with LBP (Figures 16 and 17). The implications of this phenomenon for the LPS-induced stimulation of macrophages were further investigated in Chapter 4. Additionally, SLPI was investigated in these assays and was also shown to have an inhibitory effect on the LPS-LBP interaction. Polymyxin B, used here as a positive control, was investigated in the study carried out by Scott *et al.* (2000b) and has been revealed in both studies to be a highly potent inhibitor of the interaction.

Intriguingly, while concentrations of full-length elafin at the low end of the test range effectively inhibited LPS-LBP interaction, higher concentrations (above 1.25μM) inhibited to a lesser degree and in fact appeared to enhance the binding of LPS to immobilised LBP in a dose-responsive manner (Figure 16). Investigation of this anomaly suggested that elafin mediated binding of LPS to several components of the assay, including blocking agent, plastic in the wells and even LBP itself (data not shown). It is important to note that no non-specific effects were observed other than in the presence of elafin. Two possible explanations may account for this effect of elafin at high concentrations. Firstly, it is feasible that elafin molecules may be more disposed to forming polymers at higher concentrations and this aggregation could facilitate generation of larger elafin-LPS aggregates, resulting in increased signal. Secondly, aggregates of LPS may be more effectively disassembled at higher elafin concentrations, liberating more individual LPS polymers which subsequently bind to LBP. Similar findings were obtained by Kellogg *et al.* (2001) using a monoclonal antibody (1B6) to lipid A; Kellogg's study found that 1B6 inhibition of LPS activity in the *Limulus* amoebocyte lysate (LAL) assay was decreased at higher antibody concentrations, and in this case it was the presence of more free LPS monomers which enhanced activity.

Nevertheless, in the range 0-160nM, full-length elafin effectively inhibited the LPS-LBP interaction (Figure 17). Inhibition was maximal at 160nM (approximately 45% inhibition) and generally dose-dependent below this concentration. In fact the activities of full-length elafin, SLPI, NH_2-terminus and COOH-terminus were all very similar at 156nM, whilst over the entire concentration range (0-10μM, Figures 16 and 17) the COOH-terminus was the more active of elafin's terminal domains. It is also interesting to note that the activity of the full-length molecule in inhibiting LPS-LBP binding was never as great as that of the COOH-terminal domain at any concentration, and only at lower concentrations (0-19.5nM) and intermediate concentrations (625nM-1250nM) was the full-length molecule more active than the NH_2-terminus. Despite the fact that NH_2-terminal elafin was demonstrated in Figure 7 to possess a stronger affinity for LPS than the

COOH-terminal domain, it appears that this does not correlate directly with the ability to inhibit the interaction of LPS with LBP (Figures 16 and 17).

These findings are strongly suggestive that charge plays a minor role in the inhibition of LPS-LBP interaction by the elafin peptides. Indeed, if charge were the major determinant of elafin's ability to prevent LBP binding to LPS, the full-length molecule would elicit the most potent inhibition, and the effects of the NH_2-terminal domain would be expected to be intermediate between that of the COOH-terminus and full-length elafin. In fact, polymyxin B was demonstrated to be the most effective test peptide and yet the net charge of this molecule is +5 (Schroder *et al.*, 1992), equivalent to that of elafin's NH_2-terminus and lower than that of full-length elafin. This observation, coupled with the finding that the most potent inhibition by elafin peptides was consistently educed by the moiety with the lowest cationic charge, indicates that other properties of these peptides, such as hydrophobicity, play a part in this activity.

Certainly, a number of studies have described the importance of both hydrophobicity and cationicity as determinants of affinity of endotoxin-binding molecules (Weiss *et al.*, 1983; Kellogg *et al.*, 1999; Yin *et al.*, 2003). In this regard, it may tentatively be suggested that hydrophobic residues within the COOH-terminal domain could contribute to these findings, whilst these effects may be partially masked in the full-length molecule. Although both NH_2- and COOH-terminal domains contain a number of hydrophobic residues (for example, 11 of the NH_2-domain's 50 residues are valine), the COOH-terminus contains three profoundly hydrophobic regions. These are: Ile-Ile-Leu-Ile (amino acids 56-59), Cys-Ala-Met-Leu (amino acids 61-64) and Met-Ala-Cys-Phe-Val (amino acids 89-93). The hydrophobicity of these regions of the molecule was confirmed using the online Swiss Institute of Bioinformatics ProtScale hydrophobicity scale programme (Kyte and Doolittle, 1982) (data not shown). The crystal and solution structure of the recombinant elafin molecule has revealed that the polypeptide chain folding takes a flat spiral shape composed of a flexible external region (NH_2-terminal), and a central twisted β-hairpin accompanied by two external segments linked by the proteinase

binding loop (COOH-terminal) (Tsunemi *et al.*, 1996; Francart *et al.*, 1997). Three of the four disulphide bridges connect the external segments to the central β-sheet, while the fourth disulphide bridge links the binding loop to the central β-turn (Tsunemi *et al.*, 1996). Analysis of the elafin structure, as described by these studies, reveals that the first two of the aforementioned hydrophobic regions of the COOH-terminus are exposed on the external surface of the molecule, while the third is buried within the internal core. Thus these exposed hydrophobic regions (one of which contains the Ala-Met scissile bond) may potentially play a key role in the LPS-binding activity of both full-length elafin and its COOH-terminal domain.

Expansion of the ELISA technique used to obtain the results presented in Figures 15-17 allowed a more detailed analysis of the relative affinities of elafin and LBP for LPS (Figure 18). This modified ELISA measured direct binding of elafin to LPS immobilised to polystyrene microtitre plates in complex with polymyxin B. LPS alone bound poorly to assay wells, thus the technique of pre-complexing LPS to polymyxin B in order to enhance adhesion was adapted from a study carried out by Scott and Barclay (1987). Although polymyxin B was expected to occupy binding sites on LPS required for elafin binding (Figure 14), significant binding of elafin to LPS could still be detected under the employed conditions. The concentration of polymyxin B was deemed sufficient to stabilise LPS molecules but insufficient to occupy the entire binding domain. No non-specific effects such as binding of antibodies to BSA blocking protein, as documented by Chart *et al.* (1998), were shown to contribute to signal.

Pre-incubation of elafin with LPS prior to addition to immobilised LBP appeared to be a critical step in the inhibition of LPS-LBP binding; this conclusion could be drawn from the observations that (i) LBP was capable of displacing 60% of elafin previously bound to immobilised LPS, and (ii) LBP could effectively out-compete elafin for binding to free sites within LPS when both proteins were exposed to LPS simultaneously (Figure 18). These findings further suggest that affinity of the protein-LPS interactions must rely on physicochemical factors exclusive of those involved in electrostatic interactions, given that elafin is more cationic than LBP.

It is apparent, therefore, that the affinity of LBP for LPS is stronger than that of elafin. The implications of this finding for the effects of elafin on the pro-inflammatory activity of LPS will be discussed further in the following chapter.

It seems appropriate at this point to consider whether elafin shares any structural homology with the three LPS-binding molecules LBP, BPI and polymyxin B discussed within the previous pages. Appendix III (at the end of this chapter) details an amino acid sequence alignment of the mature peptides of elafin, LBP and BPI; the signal peptide sequences have been excluded for the purposes of this alignment. Additionally the structure of polymyxin B is given. Firstly, it is clear that the sequences of LBP and BPI are highly homologous, sharing 45% identity in 456 residues (Gray *et al.*, 1989). There is very little homology in general between the sequences of LBP/BPI and that of elafin, although some similar residues do exist between the sequences and these areas are shaded. In particular a number of valine, proline, glycine and lysine residues are shared (the vast majority of which are located in the exposed external region of the elafin molecule (Tsunemi *et al.*, 1996)), for example a Val-Pro region (amino acids 5-6 of elafin) which is manifest in all three sequences. Likewise, elafin and polymyxin B share little in terms of sequence. The most important features of polymyxin B are the five positively charged α-γ-diaminobutyric acid (DAB) residues which are considered to be important for electrostatic interactions with anionic phosphate charges of lipid A, and the hydrophobic 6-methylheptanoyl/octanoyl and d-Phe-Leu regions required for interaction with fatty acid chains (Srimal *et al.*, 1996; Tsubery *et al.*, 2002).

Thus the interactions of elafin and LPS appear to take place as a result of both hydrophobic and electrostatic interactions, and in this regard it is the amphipathic nature of the elafin molecule that appears to be the key determinant of its endotoxin-binding activity.

3.4. SUMMARY

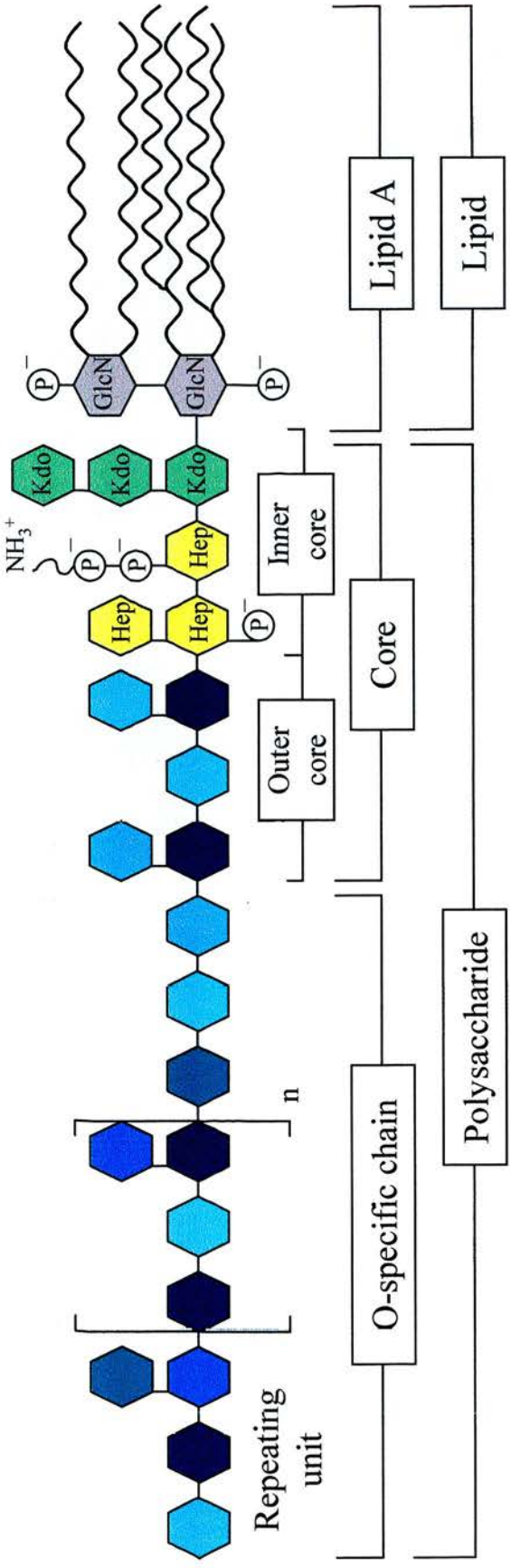
The results presented in this chapter indicate that elafin possesses LPS-binding activity, and that the binding site for elafin is located within the highly conserved lipid A portion of the LPS molecule. Moreover, binding of elafin to the lipid A domain can prevent subsequent interaction of LPS with the acute-phase glycoprotein LBP, an important process in the genesis of the inflammatory response to endotoxin. The affinity of LBP for LPS was shown, however, to be greater than that of elafin.

These findings suggest that elafin may potentially play a role in innate immunity as a modulator of host responses to LPS. The effects of elafin on LPS-mediated inflammation were further investigated in Chapter 4.

APPENDIX I

General structure of LPS from Gram-negative enterobacteria

In the depicted general scheme of smooth-form LPS architecture, the terminal O-specific chain (O-polysaccharide) may be formed by up to 50 repeating units. In the core region, an inner and outer core can be distinguished on account of the different sugars present in these domains. The lipid A domain represents the primary immunostimulatory centre of LPS, determining endotoxicity in mammalian species. Long-chain fatty acids (acyl chains) are shown attached to the disaccharide of lipid A. Each individual hexagon represents a monosaccharide subunit. Abbreviations: Hep, D-glycero-D-*manno*-heptose; Kdo, 2-keto-3-deoxyoctulosonic acid; GlcN, glucosamine; P, phosphate; NH_3^+, ethanolamine. Adapted from Haeffner-Cavaillon *et al.* (1998) and Alexander and Rietschel (2001).


GlcN
GlcN
Kdo
Kdo
Kdo
Hep
Hep
Hep
NH_3^+
P
Inner core
Outer core
Core
Lipid A
Lipid
O-specific chain
Polysaccharide
Repeating unit
n

APPENDIX II

Structures of the K12, R2 and R3 outer core oligosaccharides from the LPS of *E. coli* (A), and the chemical structure of *E. coli* lipid A (B)

A. While there are over 160 identified *E. coli* O-serotypes, only 5 unique core structures have been determined. Although all 5 share a structural theme, with a tri-hexose carbohydrate backbone and two side chain residues, the order of hexoses in the backbone and the nature, position and linkage of the side chain residues can all vary. This diagram provides the structure of K12, R2 and R3. HepII is the last residue of the inner core oligosaccharide. * indicates the point of attachment of O-antigen, but this has only been determined experimentally for R2 (diagram adapted from Amor *et al.*, 2000). **B**. The structural format of *E. coli* lipid A is widely considered to be close to that optimally recognised by human cellular LPS receptors (bisphosphorylated diglucosamine backbone substituted with 6 acyl chains) (diagram adapted from Erridge *et al.*, 2002). Abbreviations: Glc, glucose; Hep, D-glycero-D-*manno*-heptose; Gal, galactose, GlcNAc, *N*-acetyl glucosamine; Pi, phosphate.

A

E. coli K12

Glc III-1→2 -Glc II-1→3 -Glc I-1→3 -Hep II-

6 ↑ 1 (Glc III)

6 ↑ 1 (Glc I)

β-GlcNAc-1→7 -Hep IV

Gal

E. coli R2

* Glc III- 1→2 -Glc II-1→3 -Glc I-1→3 -Hep II-

2 ↑ 1 (Glc III)

6 ↑ 1 (Glc I)

GlcNAc

Gal I

E. coli R3

Glc II- 1→2 -Gal I-1→3 -Glc I-1→3 -Hep II-

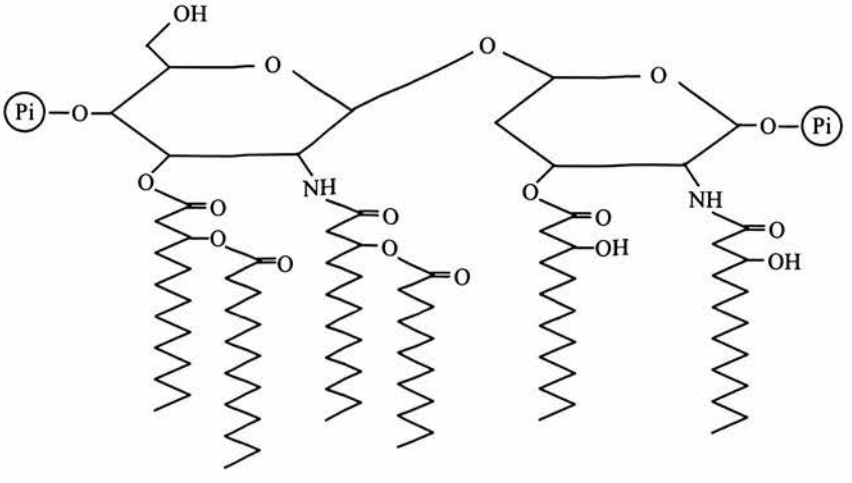
2 ↑ 1 (Glc II)

3 ↑ 1 (Gal I)

Glc III

GlcNAc

B

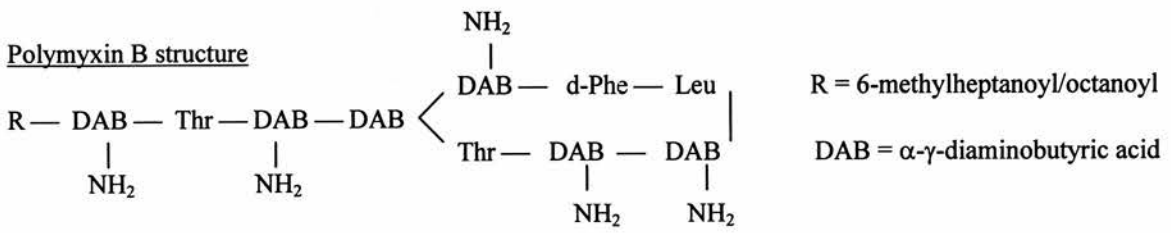


APPENDIX III

Amino acid sequence alignments of elafin, LBP and BPI and structural diagram of polymyxin B

The amino acid sequences for elafin (Sallenave and Silva, 1993), LBP and BPI (Wilde *et al.*, 1994) were aligned using the online Baylor College of Medicine ClustalW 1.8 Multiple Sequence Alignment programme. Highly homologous regions are shaded. The structure of polymyxin B was derived from Morrison and Jacobs (1976).

```
          1               15 16              30 31              45 46              60 61              75 76              90
1 LBP     ANPGLVARITDKGLQ YAAQEGLLALQSELL RITLPDFTGDLRIPH VGRGRYEFHSLNIHS CELLHSALRPVPGQG LSLSISDSSIRVQGR      90
2 BPI     VNPGVVVRISQKGLD YASQQGTAALQKELK RIKIPDYSDSFKIKH LGKGHYSFYSMDIRE FQLPSSQISMVPNVG LKFSISNANIKISGK      90
3 Elafin  ---------------- ---------------- ---------------- ---------------- ------AVTGVP--- ----VKGQ-------      10

          91              105 106             120 121             135 136             150 151             165 166             180
1 LBP     WKVRKSFFKLQGSFD VSVKGISISVNLLLG SE-SSGRPTVTASSC SSDIADVEVDMSG-D LGWLLNLFHNQIESK FQKVLESRICEMIQK    178
2 BPI     WKAQKRFLKMSGNFD LSIEGMSISADLKLG SNPTSGKPTITCSSC SSHINSVHVHISKSK VGWLIQLFHKKIESA LRNKMNSQVCEKVTN    180
3 Elafin  ----------D---- -TVKGR--------- ---------------- ---------------- ---------------- -------------V-     17

          181             195 196             210 211             225 226             240 241             255 256             270
1 LBP     SVSSDLQPYLQTLPV TTEIDSFADIDYSLV EAPRATAQMLEVMFK GEIFHRNHRSPVTLL AAVMSLPEEHNKMVY FAISDYVFNTASLVY    268
2 BPI     SVSSKLQPYFQTLPV MTKIDSVAGINYGLV APPATTAETLDVQMK GEFYSENHHNPPPFA PPVMEFPAAHDRMVY LGLSDYFFNTAGLVY    270
3 Elafin  -------PFNGQDPV KGQVS-VKGQDKVKA QEP----------VK GPVSTKPGSCPIILI RCAMLNPPNR----- ----------------     69

          271             285 286             300 301             315 316             330 331             345 346             360
1 LBP     HEEGYLNFSITDDMI PPDSNIRLTTKSFRP FVPRLARLYPNMNLE LQGSVPSAPLLNFSP GNLSVDPYMEIDAFV LLPSSSKEPVFRLSV    358
2 BPI     QEAGVLKMTLRDDMI PKESKFRLTTKFFGT FLPEVAKKFPNMKIQ IHVSASTPPHLSVQP TGLTFYPAVDVQAFA VLPNSSLASLFLIGM    360
3 Elafin  --------CLKDTDC PGIKKCCEGSCGMAC FVPQ---------- ---------------- ---------------- ----------------     95

          361             375 376             390 391             405 406             420 421             435 436             450
1 LBP     ATNVSATLTFNTSKI TGFLKPGKVKVELKE SKVGLFNAELLEALL NYYILNTFYPKFNDK LAEGFPLPLLKRVQL YDLGLQIHKDFLFLG    448
2 BPI     HTTGSMEVSAESNRL VGELKLDRLLLELKH SNIGPFPVELLQDIM NYIVPILVLPRVNEK LQKGFPLPTPARVQL YNVVLQPHQNFLLFG    450
3 Elafin  ---------------- ---------------- ---------------- ---------------- ---------------- ----------------     95

          451
1 LBP     ANVQYMRV    456
2 BPI     ADVVYK--    456
3 Elafin  --------     95
```



CHAPTER 4

THE EFFECTS OF ELAFIN PEPTIDES ON THE INFLAMMATORY RESPONSE OF MACROPHAGES AND CLARA CELLS TO LPS *IN VITRO*

4.1. AIMS

The primary aim of this chapter was to determine how the interactions of elafin with LPS, described in Chapter 3, would affect the pro-inflammatory properties of LPS *in vitro*. In the event of an effect on LPS activity being demonstrated, secondary aims were to determine which terminal domain of the elafin molecule, if either, is more active in its effects on LPS function, and to investigate whether elafin peptides themselves initiate an inflammatory response from cells *in vitro*.

Two cell types were chosen for use in this study: the murine macrophage cell line RAW 264.7, which has been extensively used by others in models of LPS-induced inflammation (for example studies performed by Gough *et al.* (1996), Ndengele *et al.* (2000) and Kiemer *et al.* (2002)); and a cell line of murine bronchiolar epithelial (Clara) cell lineage, designated DJS2-2 (Magdaleno *et al.*, 1997; Park *et al.*, 2000; Ramsay *et al.*, 2003). These cell lines represent two important cellular targets for LPS in the *in vivo* environment; indeed the response of the macrophage to LPS in particular can be considered as a critical event in the onset and progression of inflammation. Both serum-containing and serum-free conditions were investigated in order to model potential effects of elafin in varying *in vivo* compartments, for example serum-rich circulation or serum-deficient sites in the airways.

4.2. RESULTS

4.2.1. Experiments to characterise LPS response and serum requirements of RAW 264.7 murine macrophages

Several preliminary experiments were undertaken to establish a set of conditions for subsequent investigation of the effects of elafin on LPS-mediated cytokine release. These studies were carried out using the RAW 264.7 macrophage cell line, the more LPS-responsive of the two cell types. It was deemed desirable to minimise the concentration of LPS used in assays in order to avoid overwhelming the test 'system'. Additionally we wished to identify a concentration of LPS that would activate cells sufficiently in both serum-rich and serum-free conditions.

LPS-induced TNF-α secretion by RAW 264.7 macrophages was greatly enhanced in the presence of 10% foetal calf serum (Figure 1). For example, 0.5ng/ml LPS induced a mean TNF-α response of approximately 6500 pg/ml in 10% serum, compared with only 76 pg/ml in serum-free conditions; and while as little as 5 ng/ml LPS elicited secretion of 88000 pg/ml TNF-α in serum-rich conditions, the lowest LPS concentration capable of inducing significant release of cytokine in serum-free medium was 50 ng/ml (4700 pg/ml TNF-α secreted) (Figure 1). Baseline TNF-α levels were similar, although TNF-α present in serum may also have contributed slightly (17-27 pg/ml TNF-α was measured in serum-free medium compared with 59-75 pg/ml in serum-rich medium). On average, approximately 100x more LPS was required in serum-free medium to elicit a comparable response to that observed in serum-rich medium. However, most significant for these investigations was the observation that 50ng/ml LPS induced a substantial TNF-α response in both serum-free and serum-rich conditions (Figure 1). This concentration of LPS was thus used for all subsequent studies.

Considering the finding in Chapter 3 that LBP (the serum glycoprotein that plays an important role in LPS signalling by catalysing transfer of LPS monomers to CD14 (Hailman *et al.*, 1994)) has a higher LPS-binding affinity than elafin, efforts

were made to diminish the presence of this acute phase reactant in test assays. An experiment aimed at investigating the effects of LBP on LPS activity suggested that the majority of the LPS-induced activation of RAW 264.7 macrophages in serum-rich media was mediated by LBP (Figure 2). LBP is present at approximately 5-15μg/ml in human serum (Calvano *et al.*, 1994; Gallay *et al.*, 1994; Froon *et al.*, 1995). Using the figure of 5μg/ml as a reference, 25ng/ml natural human LBP added to serum-free medium reproduced the effects of the equivalent 0.5% serum on LPS stimulation of cells (Figure 2).

Since LBP was considered a potential hindrance to interaction of elafin with LPS, but was central to an enhanced cellular response to LPS, the effects of lower serum concentrations were investigated. A considerable serum effect was observed as low as 0.2% serum, and the pro-inflammatory effects of serum appeared to saturate at around 6% (Figure 3). Hence, we chose to minimise the presence of LBP in culture conditions by including 0.2% serum, approximately equivalent to 10 ng/ml (160pM) LBP.

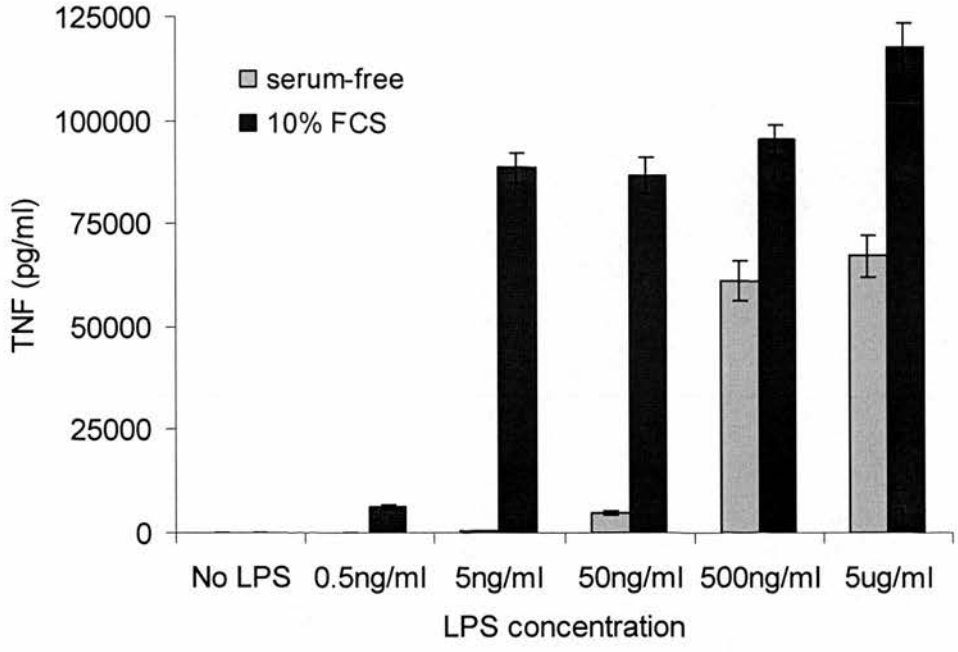


Figure 1. LPS stimulation of RAW 264.7 murine macrophages in serum-free and serum-rich conditions

RAW 264.7 murine macrophages were seeded at 5 x 10^5 cells/well of a 48-well plate overnight, washed twice with PBS and 500µl fresh medium added prior to addition of LPS. Stimulation with increasing concentrations of *E. coli* O55:B5 LPS was carried out for 4 hours at 37°C in either serum-free DMEM or DMEM containing 10% foetal calf serum (FCS). Media were removed and analysed by TNF-α ELISA. Data represent mean ± S.E. of n=3 experiments, each performed in triplicate.

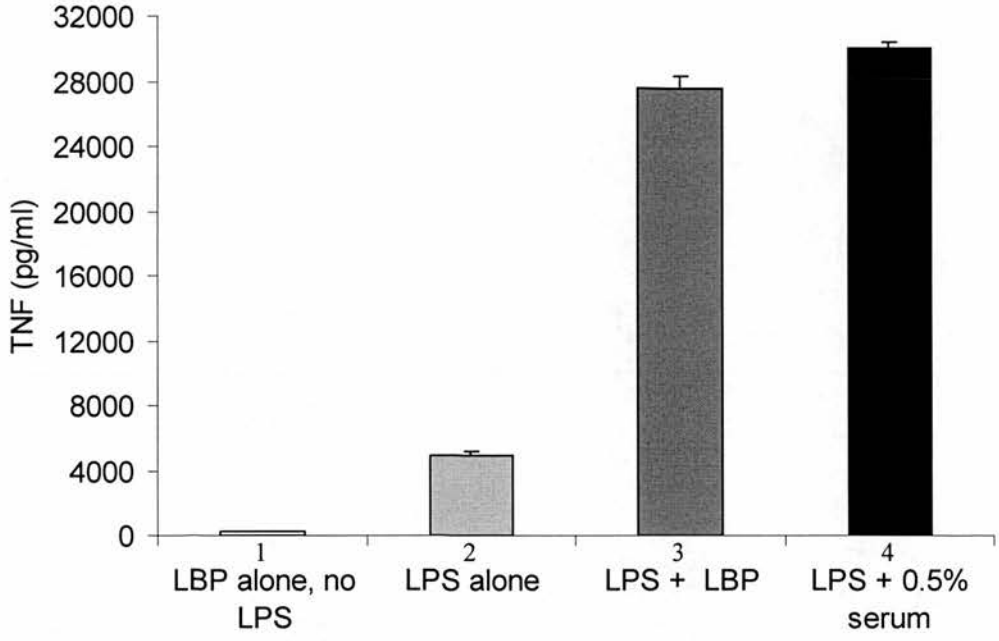


Figure 2. Natural human LBP reproduces the effects of serum on LPS-mediated stimulation of RAW 264.7 murine macrophages

RAW 264.7 murine macrophages were seeded overnight at 5 x 10^5 cells/well of a 48-well plate and washed twice with PBS. 500µl fresh DMEM was added, containing: no FCS (Bars 1, 2 and 3); 0.5% FCS (Bar 4); or 25ng/ml natural human LBP (approximately equivalent to the concentration present in 0.5% serum) (Bars 1 and 3). 50ng/ml *E. coli* O55:B5 LPS was incubated with cells for 4 hours at 37°C. Media were removed and analysed by TNF-α ELISA. Data are derived from a single experiment, carried out in triplicate. Values represent mean ± SD.

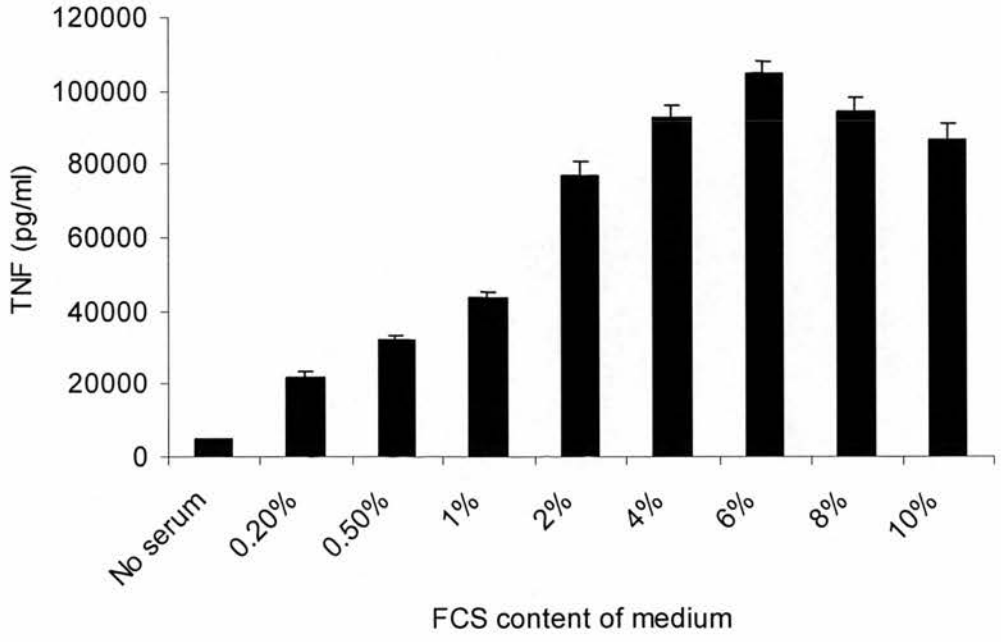


Figure 3. TNF-α response of RAW 264.7 murine macrophages to 50ng/ml LPS is enhanced by FCS concentrations as low as 0.2%.

RAW 264.7 murine macrophages were seeded overnight at 5 x 10^5 cells/ well of a 48-well plate, washed twice with PBS and 500µl of fresh DMEM containing varying concentrations of FCS added prior to LPS stimulation. 50ng/ml *E. coli* O55:B5 LPS was incubated with cells for 4 hours at 37°C, and media analysed by TNF ELISA. Data represent the mean ± S.E. of n=3 experiments, each performed in triplicate.

4.2.2. The effects of elafin peptides on LPS-mediated TNF-α secretion by RAW 264.7 murine macrophages

4.2.2.1. LPS stimulation of macrophages in medium containing serum

Prior to investigating the effects of elafin on the TNF-α response of macrophages to LPS, it was important to determine whether elafin itself induced the cellular release of TNF-α. This investigation was also of consequence since it is known that similar defensin and antimicrobial peptide molecules can be cytotoxic to mammalian cells, albeit at high concentrations in the μM range (compared to the nM concentrations of elafin used here) (Lehrer *et al.*, 1993; Risso *et al.*, 1998). No significant TNF-α secretion was induced by elafin peptides (full-length molecule, NH_2-terminal domain or COOH-terminal domain) (Figure 4). Additionally, incubation of elafin peptides with murine macrophages was not associated with morphological damage to the cellular monolayer, as evidenced by light microscopy, and trypan blue exclusion indicated that elafin peptides induced no apparent damage to cells (data not shown).

Stimulation of RAW 264.7 macrophages with LPS in medium containing 0.2% serum induced a typical TNF-α release of between 20000-30000pg/ml (20-30ng/ml) over the 4-hour incubation time, compared with a TNF-α secretion of approximately 70-150pg/ml by unstimulated cells. Addition of full-length elafin to cell culture medium at the same time as LPS had no significant effects on the LPS-induced release of TNF-α (Figure 5A). However, incubating LPS with full-length elafin for 30 minutes at 37°C prior to addition to cells reduced the secretion of TNF-α, and inhibition of LPS-induced TNF-α release was significant at 10nM and 100nM full-length elafin (Figure 5B). The maximum inhibition observed was around 40% at 100nM elafin.

Similar assays were undertaken in order to investigate the contribution of NH_2- and COOH-terminal elafin domains to the anti-inflammatory activity of the full-length molecule. The NH_2-terminal domain had no significant effect on LPS-

mediated TNF-α release from macrophages, even when incubated with LPS prior to addition to cells (Figure 6). A trend towards decreased TNF-α response to LPS that had been pre-incubated with NH_2-terminus was observed, but the down-regulation of cellular response was not significant (Figure 6B). However, the COOH-terminal domain significantly inhibited TNF-α secretion but, as was the case with the full-length molecule, this inhibition was observed only when LPS had been incubated with COOH-terminus prior to addition to test wells and not on simultaneous addition of LPS and peptide (Figure 7). Inhibition of TNF-α was maximal at 100nM COOH-terminus and the degree of inhibition, approximately 34% at this concentration of peptide, was very similar to that effected by the full-length molecule (40% as per Figure 5B). These observations suggest that the majority of elafin's LPS-inhibitory activity in serum-containing milieu is contributed by its COOH-terminal domain.

During the course of these investigations, difficulties were again encountered in finding a suitable negative control peptide for the LPS-induced activation of cells, as discussed in Chapter 3. In early investigations using macrophages as a model for LPS-mediated inflammation, polymyxin B was used as a positive control for inhibition of TNF-α secretion and this peptide elicited reproducible, significant down-regulation of TNF-α (not shown). However, identifying a 'passive' peptide which had no effects on LPS activation proved difficult. Cytochrome c, which we had previously shown could bind to LPS and inhibit LPS-LBP interaction, also effectively down-regulated LPS-mediated TNF-α secretion by macrophages (data not shown). Similarly, protamine was found to inhibit TNF-α secretion (not shown). BSA, which could bind LPS and inhibit interaction of LPS with LBP during the investigations described in Chapter 3, was shown to have a slight anti-inflammatory effect on LPS-mediated stimulation over the test concentrations used here. The terminal domains of elafin have thus been used in these studies as 'internal' controls. It could also be suggested that control peptides are perhaps not as important in assays in which elafin is added simultaneously with LPS in serum, since in this case a range of protein molecules are present in the test system.

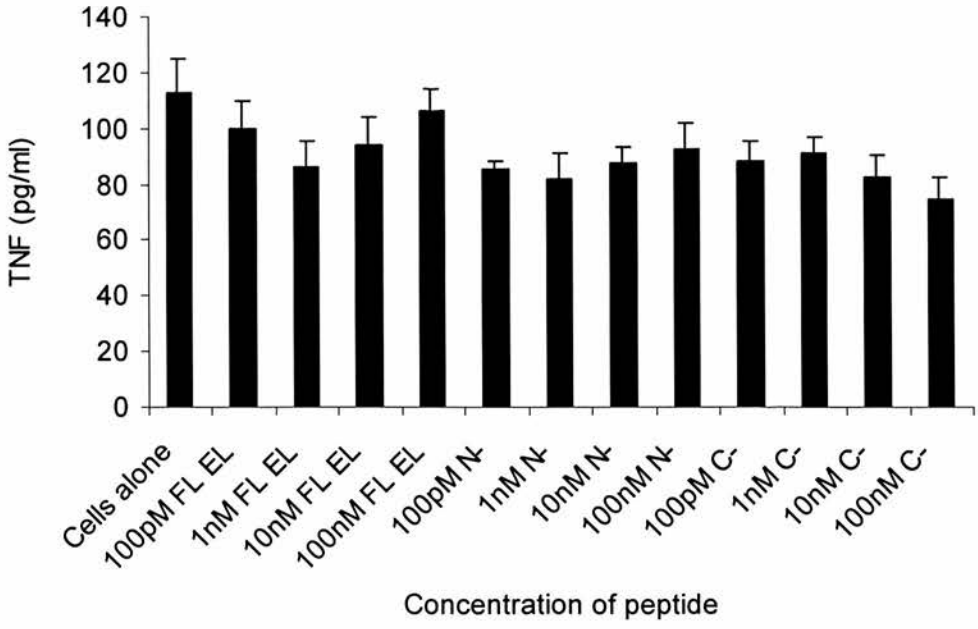


Figure 4. Elafin peptides have no significant effect on secretion of TNF-α by RAW 264.7 murine macrophages (medium containing serum)

RAW 264.7 murine macrophages were seeded at 5 x 10^5 cells/well of a 48-well plate overnight, washed twice with PBS and 500µl DMEM containing 0.2% FCS added. Full-length elafin (FL EL), NH_2-terminal elafin domain (N-) or COOH-terminal elafin (C-) were incubated with cells for 4 hours, and media were analysed by TNF-α ELISA. Values represent mean ± S.E. of n=4 experiments, each performed in triplicate.

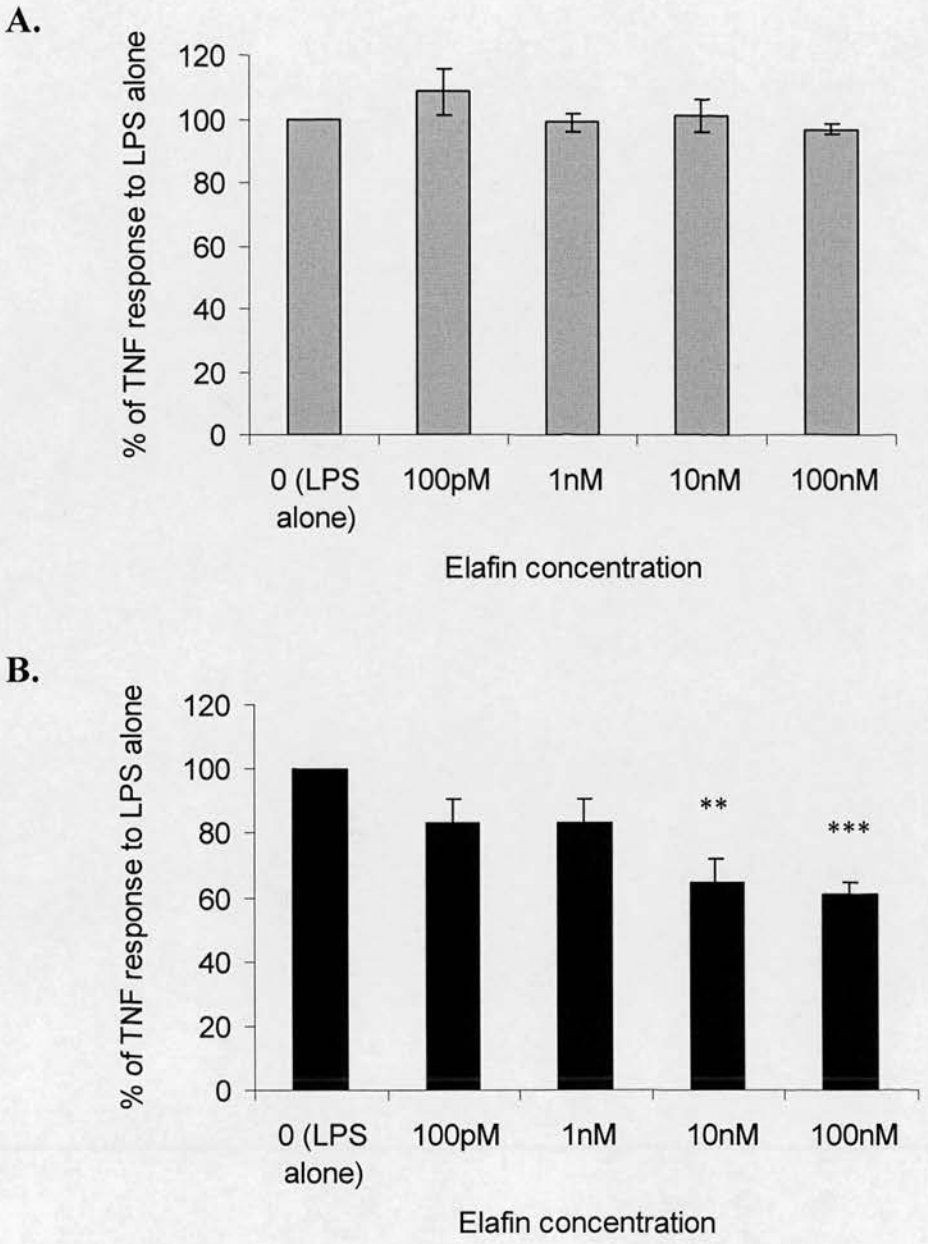


Figure 5. Full-length elafin down-regulates LPS-mediated TNF-α secretion by RAW 264.7 murine macrophages, in medium containing serum

RAW 264.7 murine macrophages were seeded at 5 x 10^5 cells/well of a 48-well plate overnight, washed twice with PBS and 500µl DMEM containing 0.2% FCS added. 50ng/ml *E. coli* O55:B5 LPS was incubated with cells for 4 hours either alone or in the presence of increasing concentrations of elafin; media were analysed by TNF-α ELISA. GRAPH A: Elafin and LPS added to cells simultaneously. GRAPH B: Elafin pre-incubated with LPS for 30 mins at 37°C prior to addition to cells. Values represent mean ± S.E. of n=5 (A) or n=6 (B) experiments, each performed in triplicate. ** = significant difference, $P<0.01$, compared with 'LPS alone'. *** = significant difference, $P<0.001$, compared with 'LPS alone'.

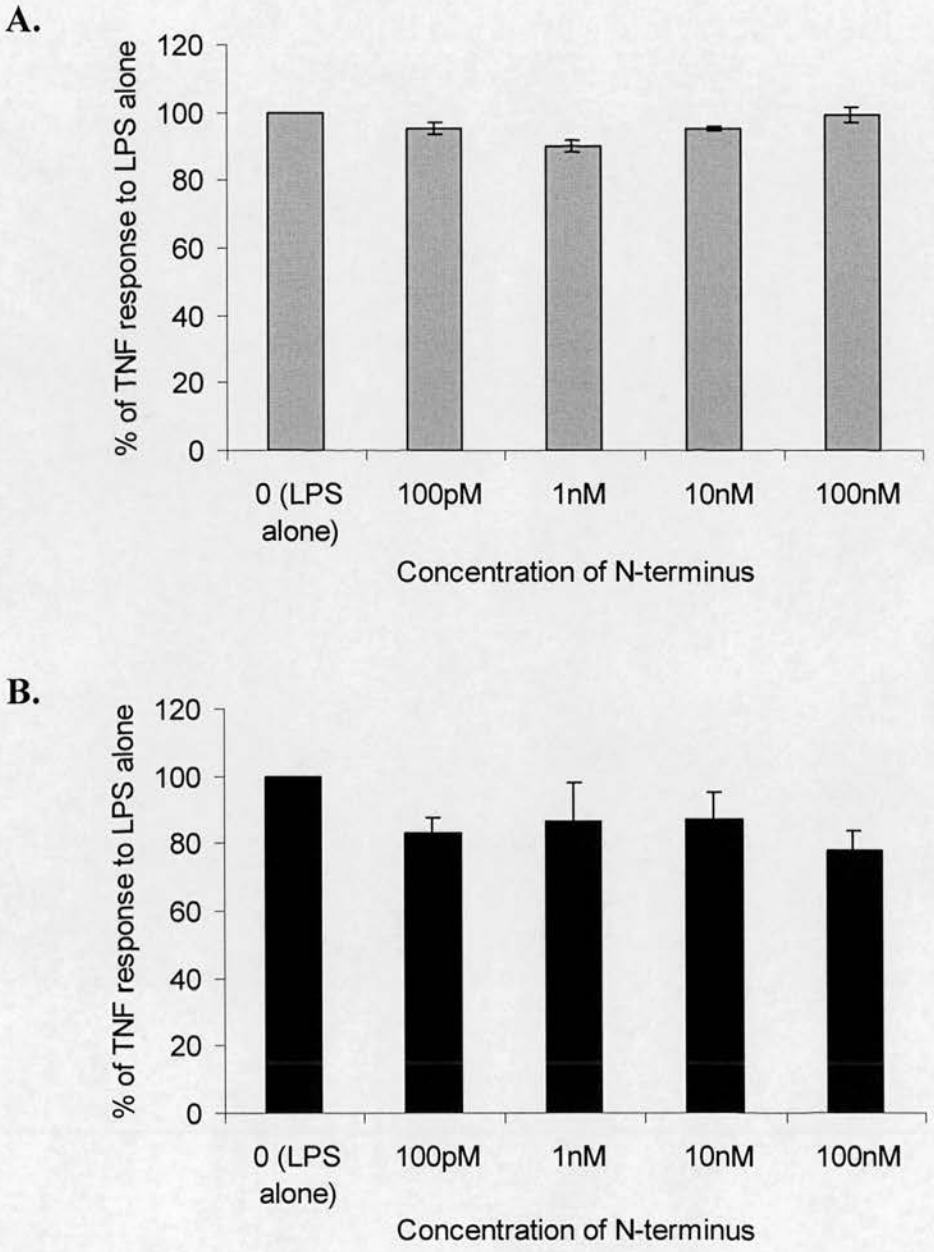


Figure 6. N-terminal elafin domain has no significant effect on LPS-mediated TNF-α secretion by RAW 264.7 murine macrophages, in medium containing serum

RAW 264.7 murine macrophages were seeded at 5 x 10^5 cells/well of a 48-well plate overnight, washed twice with PBS and 500µl DMEM containing 0.2% FCS added. 50ng/ml *E. coli* O55:B5 LPS was incubated with cells for 4 hours either alone or in the presence of increasing concentrations of NH_2-terminus; media were analysed by TNF-α ELISA. GRAPH A: NH_2-terminus and LPS added to cells simultaneously. GRAPH B: NH_2-terminus pre-incubated with LPS for 30 mins at 37°C prior to addition to cells. Values represent mean ± S.E. of n=3 (A) or n=4 (B) experiments, each performed in triplicate.

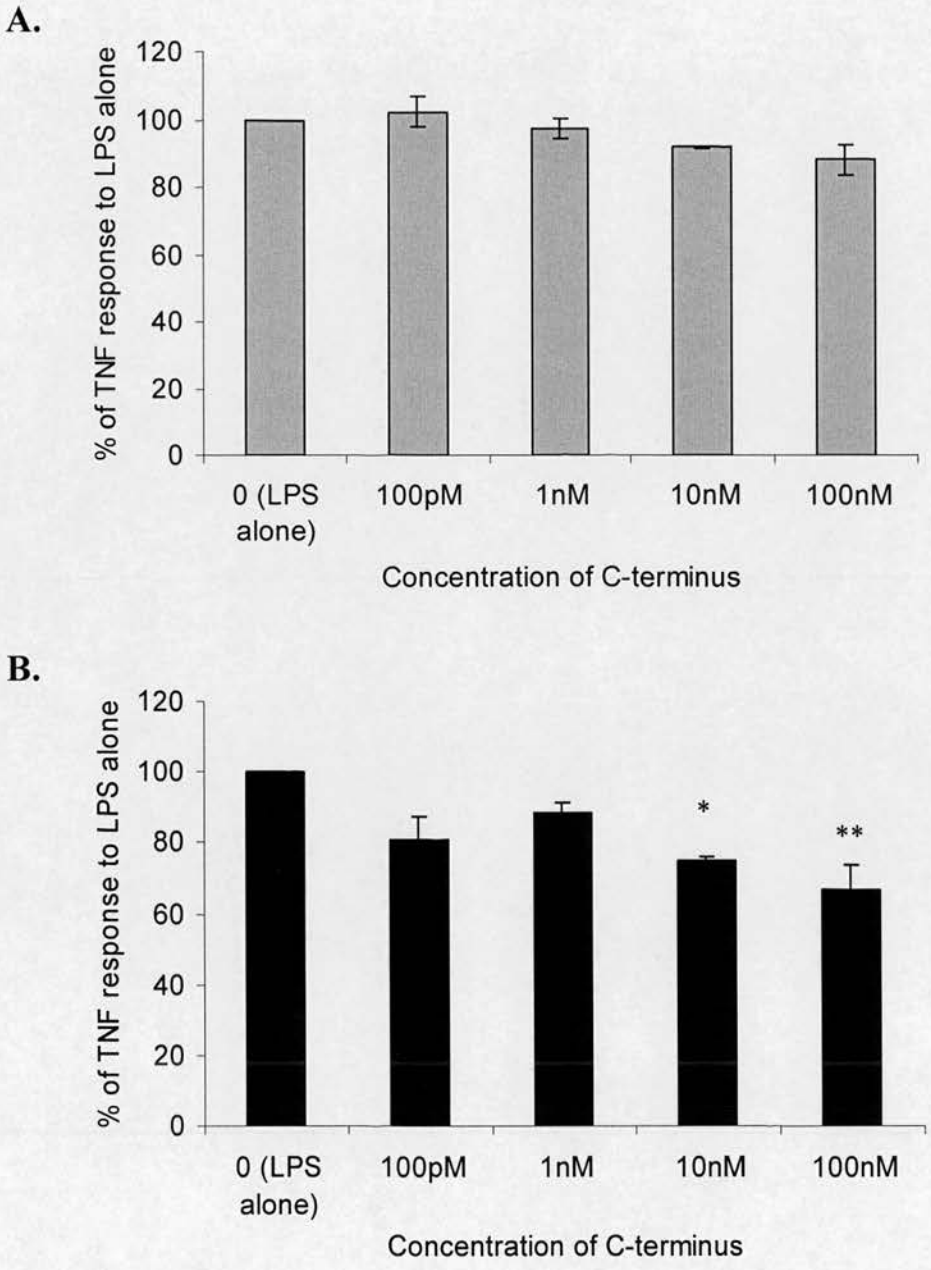


Figure 7. C-terminal elafin domain down-regulates LPS-mediated TNF-α secretion by RAW 264.7 murine macrophages, in medium containing serum

RAW 264.7 murine macrophages were seeded at 5 x 10^5 cells/well of a 48-well plate overnight, washed twice with PBS and 500µl DMEM containing 0.2% FCS added. 50ng/ml *E. coli* O55:B5 LPS was incubated with cells for 4 hours either alone or in the presence of increasing concentrations of COOH-terminus; media were analysed by TNF-α ELISA. GRAPH A - COOH-terminus and LPS added to cells simultaneously. GRAPH B – COOH-terminus pre-incubated with LPS for 30 mins at 37°C prior to addition to cells. Values represent mean ± S.E. of n=3 experiments, each performed in triplicate. * = significant difference, $P<0.05$, compared with 'LPS alone'. ** = significant difference, $P<0.01$, compared with 'LPS alone'.

4.2.2.2. LPS stimulation of macrophages in serum-free medium

Prior to commencing investigation of the effects of elafin on LPS stimulation of macrophages in serum-free milieu, it was again deemed necessary to study the effects of elafin peptides alone; this is especially relevant in the case of serum-free conditions since serum has been shown to protect cells from the cytotoxic effects of defensins (Lehrer *et al.*, 1993). Elafin peptides themselves elicited no significant release of TNF-α by murine macrophages, as compared with cells incubated in the absence of peptides (Figure 8). As was previously demonstrated in medium containing serum, elafin peptides also caused no discernible damage to the cell monolayer when incubated with cells in serum-free medium, as assessed by light microscopy and trypan blue exclusion (not shown).

Although the magnitude of macrophage response to LPS in serum-free medium was much lower than that elicited in medium containing serum, secretion of TNF-α was still considerable. Typical values obtained on analysis of media from LPS-stimulated cells were between 3500-5500 pg/ml TNF-α, as compared with TNF-α levels of around 8-60 pg/ml when cells were incubated for 4 hours in the absence of LPS.

Interestingly, the effects of elafin on LPS-mediated stimulation of macrophages in serum-free conditions were in stark contrast to those previously observed in serum-containing conditions. Although full-length elafin had no effect on LPS-induced TNF release when LPS and elafin were added simultaneously, pre-incubating elafin with LPS significantly enhanced the pro-inflammatory activity of LPS (Figure 9). The effects of elafin were again maximal in the range 10nM-100nM, and these concentrations enhanced the TNF-α response of macrophages by up to 300%.

Both the NH_2- and the COOH-terminal domains of the full-length molecule were shown to contribute to this pro-inflammatory activity (Figures 10 and 11). Unlike full-length elafin, neither terminus induced any significant effects at 10nM,

but both domains significantly enhanced LPS-induced TNF-α secretion at 100nM. As was observed in the previous section using serum-containing milieu, the COOH-terminus exerted a greater activity than the NH_2-terminus. Indeed, while both the full-length molecule and the NH_2-terminal domain enhanced TNF-α secretion by up to approximately 300% (Figures 9 and 10), the COOH-terminal domain up-regulated TNF-α secretion by 600% at 100nM; this suggests that elafin's terminal domains do not function synergistically, and that the observed pro-inflammatory activity of the full-length molecule may be regulated by an interaction between its constituent domains.

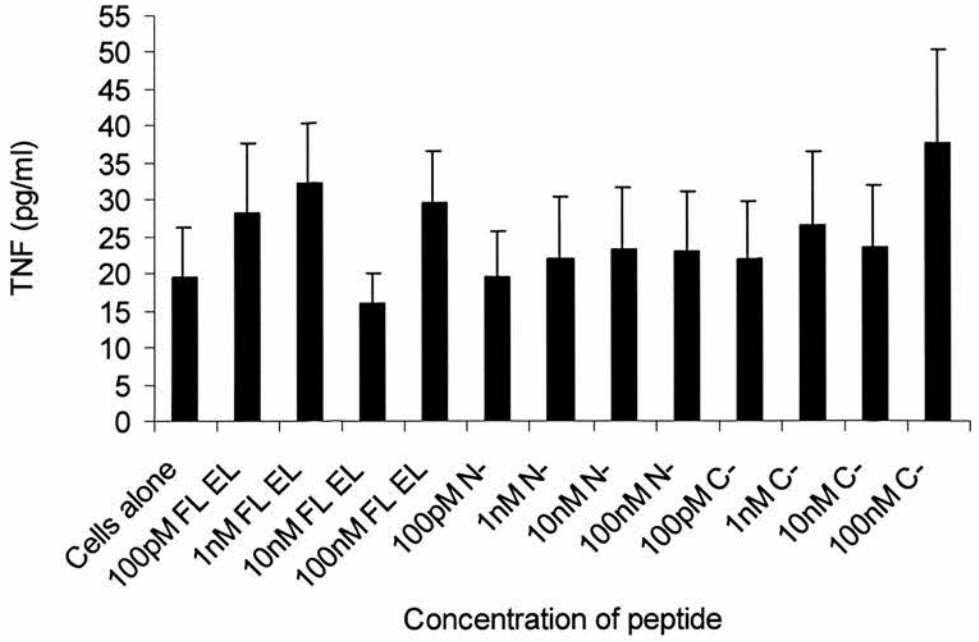


Figure 8. Elafin peptides have no significant effect on secretion of TNF-α by RAW 264.7 murine macrophages (serum-free medium)

RAW 264.7 murine macrophages were seeded at 5 x 10^5 cells/well of a 48-well plate overnight, washed twice with PBS and 500µl serum-free DMEM added. Full-length elafin (FL EL), NH_2-terminal elafin domain (N-) or COOH-terminal elafin (C-) were incubated with cells for 4 hours, and media were analysed by TNF-α ELISA. Values represent mean ± S.E. of n=4 experiments, each performed in triplicate.

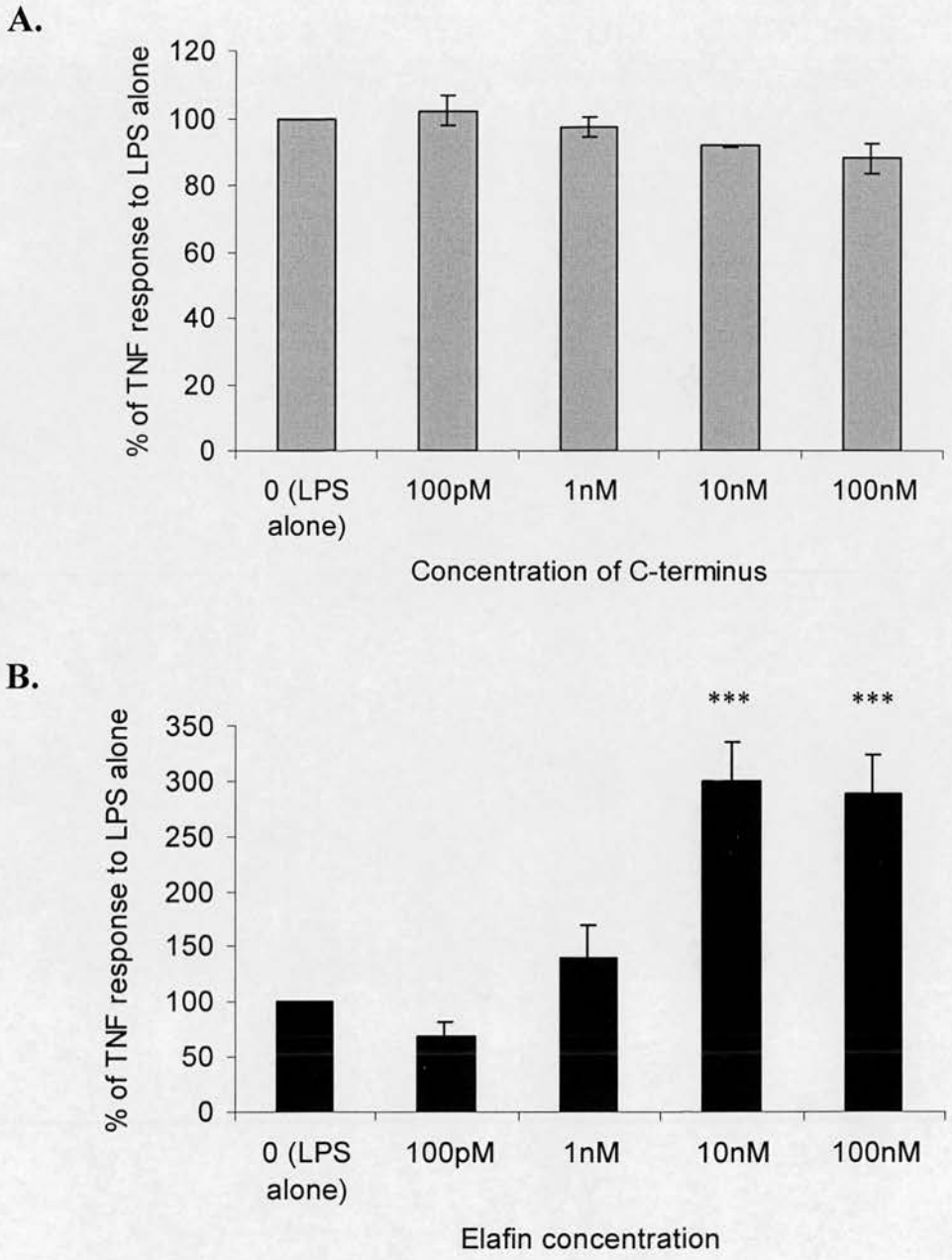


Figure 9. Full-length elafin enhances LPS-mediated TNF-α secretion by RAW 264.7 murine macrophages, in serum-free medium

RAW 264.7 murine macrophages were seeded at 5 x 10^5 cells/well of a 48-well plate overnight, washed twice with PBS and 500µl serum-free DMEM added. 50ng/ml *E. coli* O55:B5 LPS was incubated with cells for 4 hours either alone or in the presence of increasing concentrations of elafin; media were analysed by TNF-α ELISA. GRAPH A: Elafin and LPS added to cells simultaneously. GRAPH B: Elafin pre-incubated with LPS for 30 mins at 37°C prior to addition to cells. Values represent mean ± S.E. of n=5 (A) or n =4 (B) experiments, each performed in triplicate. *** = significant difference, $P<0.001$, compared with 'LPS alone'.

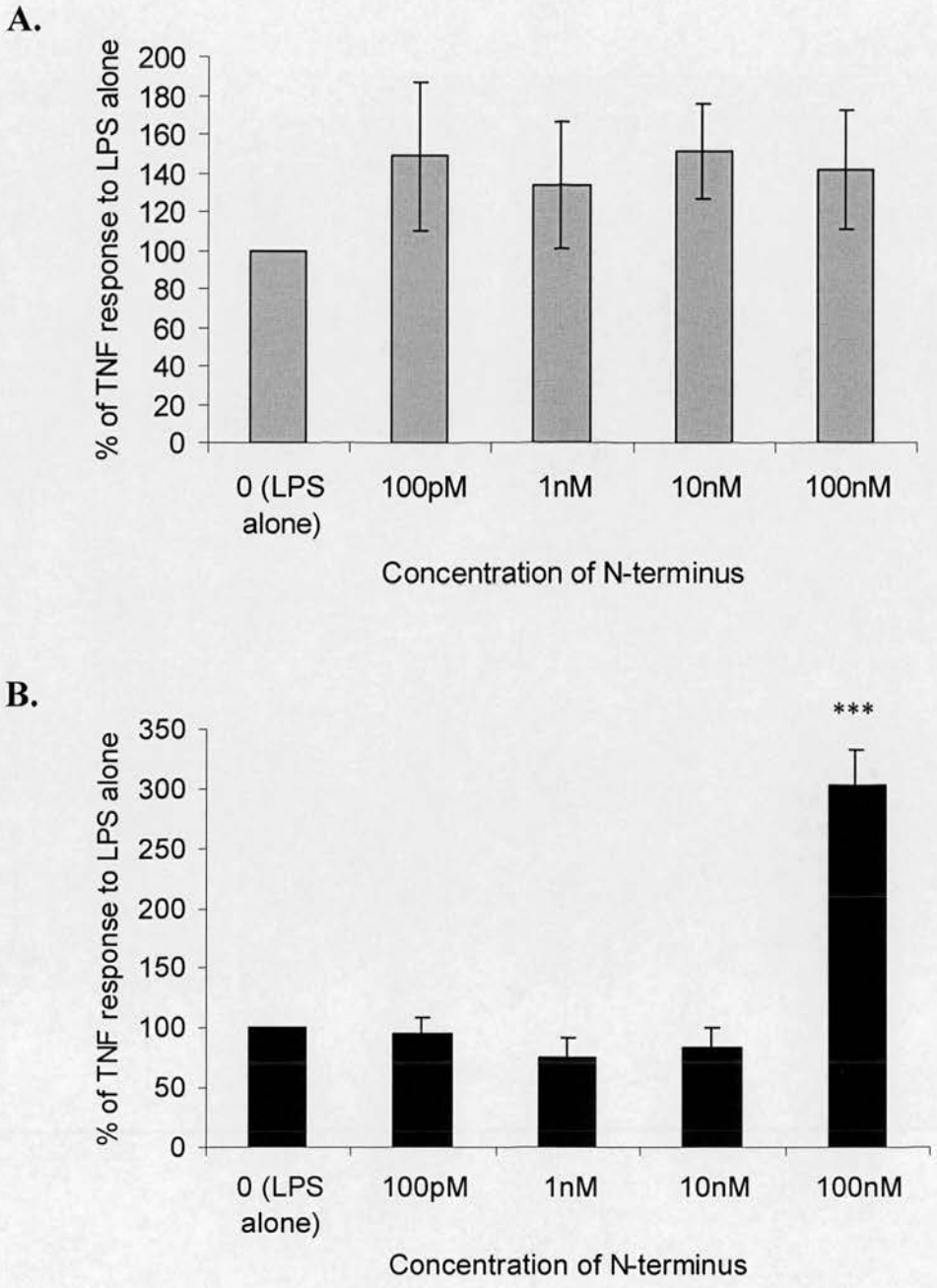


Figure 10. N-terminal elafin domain enhances LPS-mediated TNF-α secretion by RAW 264.7 murine macrophages, in serum-free medium

RAW 264.7 murine macrophages were seeded at 5 x 10^5 cells/well of a 48-well plate overnight, washed twice with PBS and 500µl serum-free DMEM added. 50ng/ml *E. coli* O55:B5 LPS was incubated with cells for 4 hours either alone or in the presence of increasing concentrations of NH_2-terminus; media were analysed by TNF-α ELISA. GRAPH A: NH_2-terminus and LPS added to cells simultaneously. GRAPH B: NH_2-terminus pre-incubated with LPS for 30 mins at 37°C prior to addition to cells. Values represent mean ± S.E. of n=4 experiments, each performed in triplicate. *** = significant difference, $P<0.001$, compared with 'LPS alone'.

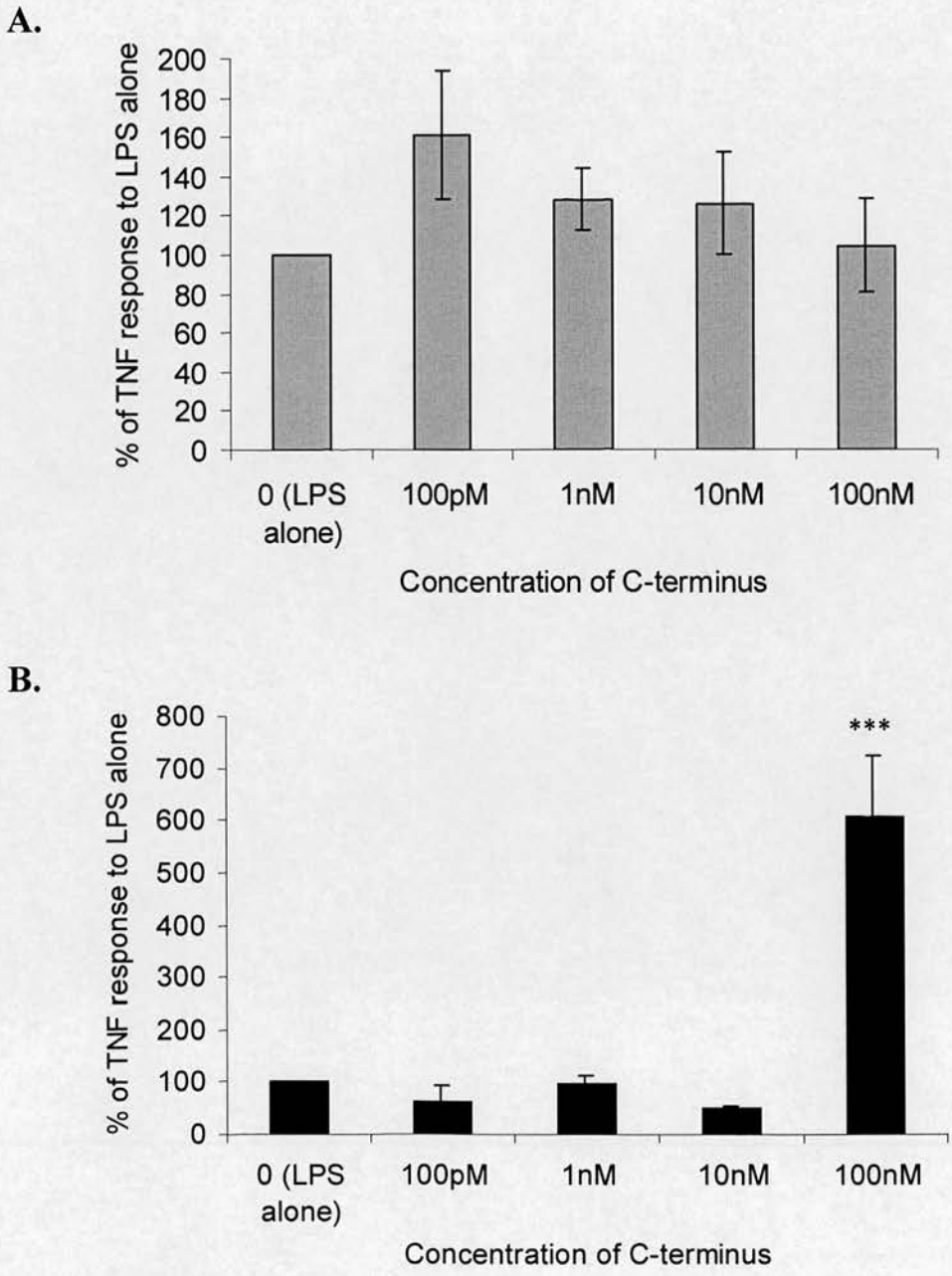


Figure 11. C-terminal elafin domain enhances LPS-mediated TNF-α secretion by RAW 264.7 murine macrophages, in serum-free medium

RAW 264.7 murine macrophages were seeded at 5 x 10^5 cells/well of a 48-well plate overnight, washed twice with PBS and 500µl serum-free DMEM added. 50ng/ml *E. coli* O55:B5 LPS was incubated with cells for 4 hours either alone or in the presence of increasing concentrations of COOH-terminus; media were analysed by TNF-α ELISA. GRAPH A - COOH-terminus and LPS added to cells simultaneously. GRAPH B – COOH-terminus pre-incubated with LPS for 30 mins at 37°C prior to addition to cells. Values represent mean ± S.E. of n=4 (A) or n=3 (B) experiments, each performed in triplicate. *** = significant difference, $P<0.001$, compared with 'LPS alone'.

4.2.3. The effects of elafin peptides on LPS-mediated MIP-2 secretion by murine bronchiolar epithelial cells (Clara cells)

4.2.3.1. LPS stimulation of Clara cells in medium containing serum

Although Clara cells are unlikely to be bathed in serum-containing milieu in airways of healthy individuals *in vivo*, respiratory inflammation or injury can lead to increased epithelial permeability and 'leakage' of serum into the lung (Arsalane *et al.*, 2000; Broeckaert *et al.*, 2000). With this in mind, the effects of elafin on the LPS stimulation of Clara cells were investigated in both serum-free and serum-containing conditions. Moreover, the C-X-C chemokine MIP-2 (a homologue of human IL-8) was chosen as a marker of LPS-induced Clara cell activation since, in contrast to macrophages, epithelial cells produce very little TNF-α (Mannel and Echtenacher, 2000).

Baseline levels of MIP-2 produced by Clara cells were low (approximately 8-12 pg/ml) and elafin peptides (full-length, NH_2-terminus and COOH-terminus) had no significant effect on MIP-2 secretion (Figure 12). Moreover, incubation of elafin peptides was not associated with any morphological perturbation of the Clara cell monolayer or cellular injury, as evidenced by light microscopy and trypan blue exclusion (not shown).

Stimulation of Clara cells with LPS typically resulted in a secretion of between 175-275 pg/ml MIP-2. Addition of full-length elafin to cells at the same time as LPS had no effect on the MIP-2 secretion by Clara cells, however pre-incubating elafin with LPS before adding to culture media caused a significant enhancement in the cellular response (Figure 13). The effects of elafin were maximal at 10nM-100nM, and at these concentrations MIP-2 secretion was around 200% of that observed in response to LPS alone.

NH_2-terminal and COOH-terminal elafin domains also had no effect on cellular response to LPS when LPS and peptides were added concurrently (Figures 14A and 15A respectively). However, following pre-incubation with LPS both

domains enhanced MIP-2 secretion to a significant degree at 100nM peptide (Figures 14B and 15B). The COOH-terminal domain was shown to be the more active domain since it up-regulated LPS-induced MIP-2 release by 240%, compared with a 140% increase in the case of the NH_2-terminal domain.

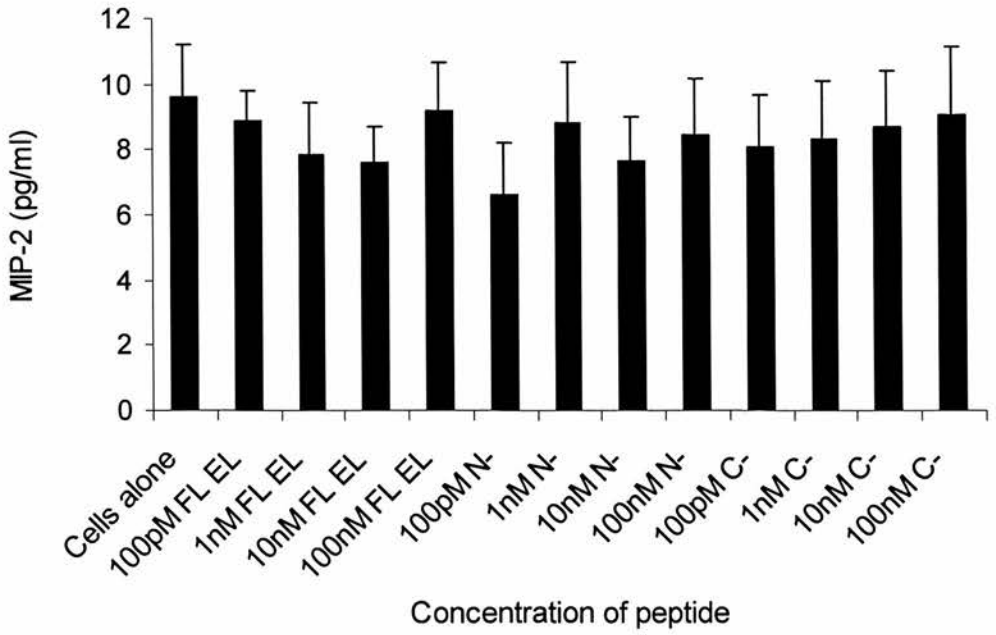


Figure 12. Elafin peptides have no significant effect on secretion of MIP-2 by murine Clara cells (medium containing serum)

Clara cells were seeded at 3 x 10^5 cells/well of a 48-well plate overnight, washed twice with PBS and 500µl DMEM containing 0.2% FCS added. Full-length elafin (FL EL), NH_2-terminal elafin domain (N-) or COOH-terminal elafin (C-) were incubated with cells for 4 hours, and media were analysed by MIP-2 ELISA. Values represent mean ± S.E. of n=3 experiments, each performed in triplicate.

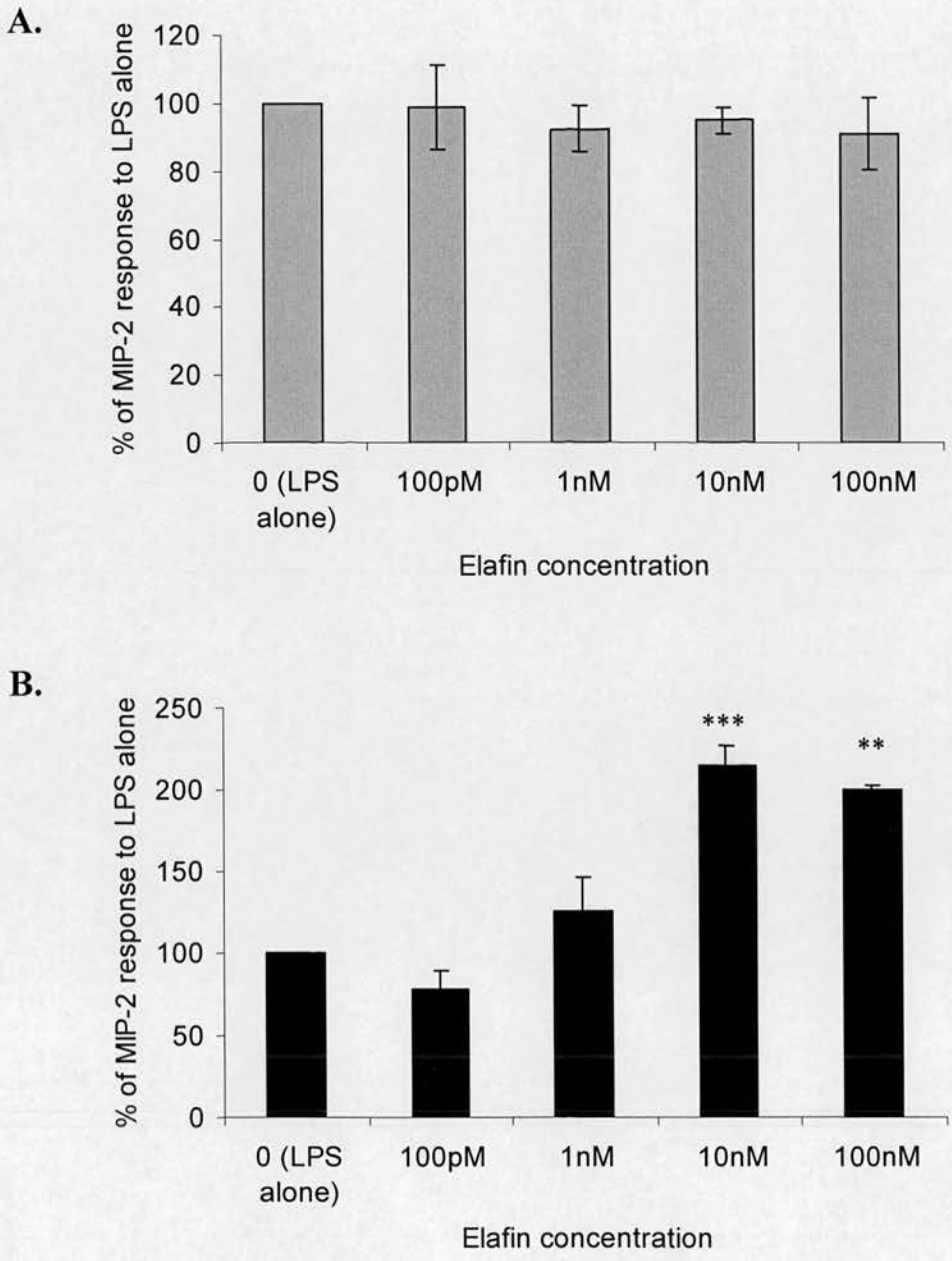


Figure 13. Full-length elafin enhances LPS-mediated MIP-2 secretion by murine Clara cells, in medium containing serum

Clara cells were seeded at 3 x 10^5 cells/well of a 48-well plate overnight, washed twice with PBS and 500µl DMEM containing 0.2% FCS added. 50ng/ml *E. coli* O55:B5 LPS was incubated with cells for 4 hours either alone or in the presence of increasing concentrations of elafin; media were analysed by MIP-2 ELISA. GRAPH A: Elafin and LPS added to cells simultaneously. GRAPH B: Elafin pre-incubated with LPS for 30 mins at 37°C prior to addition to cells. Values represent mean ± S.E. of n=4 (A) or n=3 (B) experiments, each performed in triplicate. ** = significant difference, $P<0.01$, compared with 'LPS alone'. *** = significant difference, $P<0.001$, compared with 'LPS alone'.

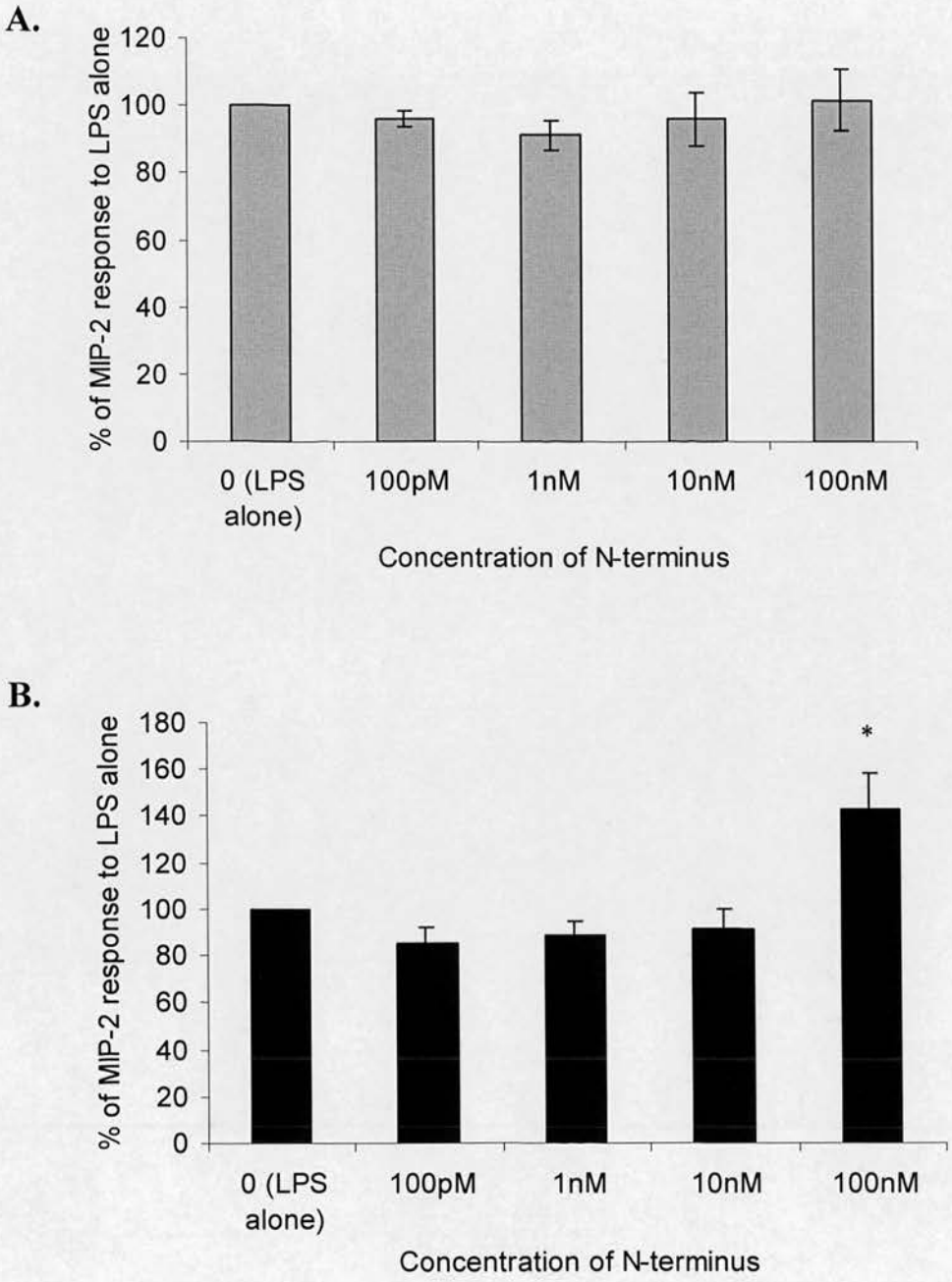


Figure 14. N-terminal elafin domain enhances LPS-mediated MIP-2 secretion by murine Clara cells, in medium containing serum

Clara cells were seeded at 3 x 10^5 cells/well of a 48-well plate overnight, washed twice with PBS and 500µl DMEM containing 0.2% FCS added. 50ng/ml *E. coli* O55:B5 LPS was incubated with cells for 4 hours either alone or in the presence of increasing concentrations of NH_2-terminus; media were analysed by MIP-2 ELISA. GRAPH A: NH_2-terminus and LPS added to cells simultaneously. GRAPH B: NH_2-terminus pre-incubated with LPS for 30 mins at 37°C prior to addition to cells. Values represent mean ± S.E. of n=4 experiments, each performed in triplicate. * = significant difference, $P<0.05$, compared with 'LPS alone'.

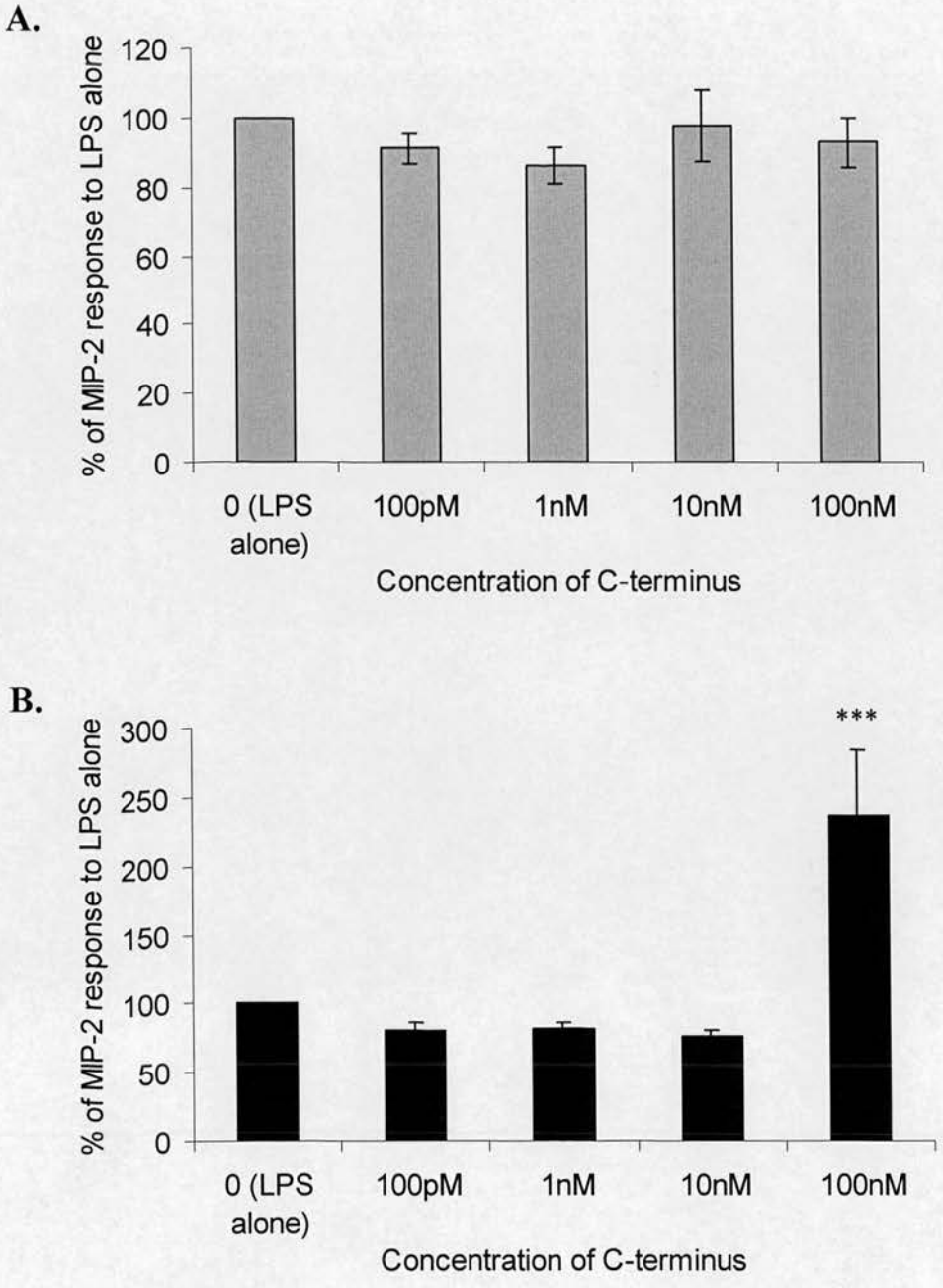


Figure 15. C-terminal elafin domain enhances LPS-mediated MIP-2 secretion by murine Clara cells, in medium containing serum

Clara cells were seeded at 3 x 10^5 cells/well of a 48-well plate overnight, washed twice with PBS and 500µl DMEM containing 0.2% FCS added. 50ng/ml *E. coli* O55:B5 LPS was incubated with cells for 4 hours either alone or in the presence of increasing concentrations of COOH-terminus; media were analysed by MIP-2 ELISA. GRAPH A - COOH-terminus and LPS added to cells simultaneously. GRAPH B – COOH-terminus pre-incubated with LPS for 30 mins at 37°C prior to addition to cells. Values represent mean ± S.E. of n=4 experiments, each performed in triplicate. *** = significant difference, $P<0.001$, compared with 'LPS alone'.

4.2.3.2. LPS stimulation of Clara cells in serum-free medium

As in previous sections in this chapter the effects of elafin peptides on cells were firstly assessed, independently of LPS-stimulation assays. Full-length elafin and its terminal domains did not themselves induce significant MIP-2 secretion by Clara cells (Figure 16) and did not cause damage to the cell monolayer (not shown).

In serum-free medium, a larger concentration of LPS was required to elicit a response from Clara cells than in serum-containing conditions (1μg/ml LPS as compared to 50ng/ml LPS in the previous section). Clara cells typically responded to stimulation with 1μg/ml LPS in serum-free medium with secretion of MIP-2 levels between 80-250 pg/ml.

In these conditions, full-length elafin had no effect on the LPS-mediated release of MIP-2, when added either simultaneously with LPS or following pre-incubation with LPS (Figure 17). There was a trend towards an enhancement of cellular chemokine secretion in response to LPS that had been pre-incubated with elafin, but this trend was found to be non-significant (Figure 17B).

Similar findings were obtained on investigation of the effects of the NH_2-terminal domain on LPS-mediated MIP-2 release (Figure 18); cells appeared to be more responsive to LPS that had been pre-incubated with NH_2-terminus, but this did not reach statistical significance (Figure 18B).

The COOH-terminal domain, on the other hand, up-regulated LPS-mediated MIP-2 release at all concentrations tested and this effect was observed even when peptides were added to cell culture medium at the same time as LPS (Figure 19). The maximal enhancement of LPS stimulation was 215%, on incubation of LPS with 1nM COOH-terminus prior to addition to cells (Figure 19B). These results are again suggestive of a prominent role for the COOH-terminal domain in the LPS-binding activities of the elafin molecule, although this effect may be masked or modulated to some extent in the full-length peptide.

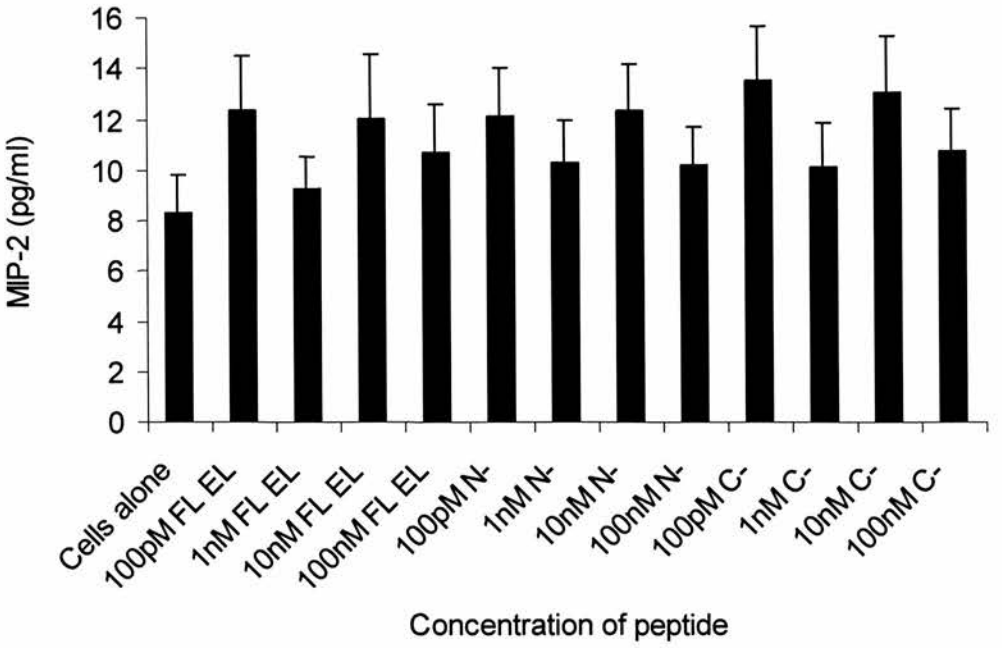


Figure 16. Elafin peptides have no significant effect on secretion of MIP-2 by murine Clara cells (serum-free medium)

Clara cells were seeded at 3 x 10^5 cells/well of a 48-well plate overnight, washed twice with PBS and 500µl serum-free DMEM added. Full-length elafin (FL EL), NH_2-terminal elafin domain (N-) or COOH-terminal elafin (C-) were incubated with cells for 4 hours, and media were analysed by MIP-2 ELISA. Values represent mean ± S.E. of n=5 experiments, each performed in triplicate.

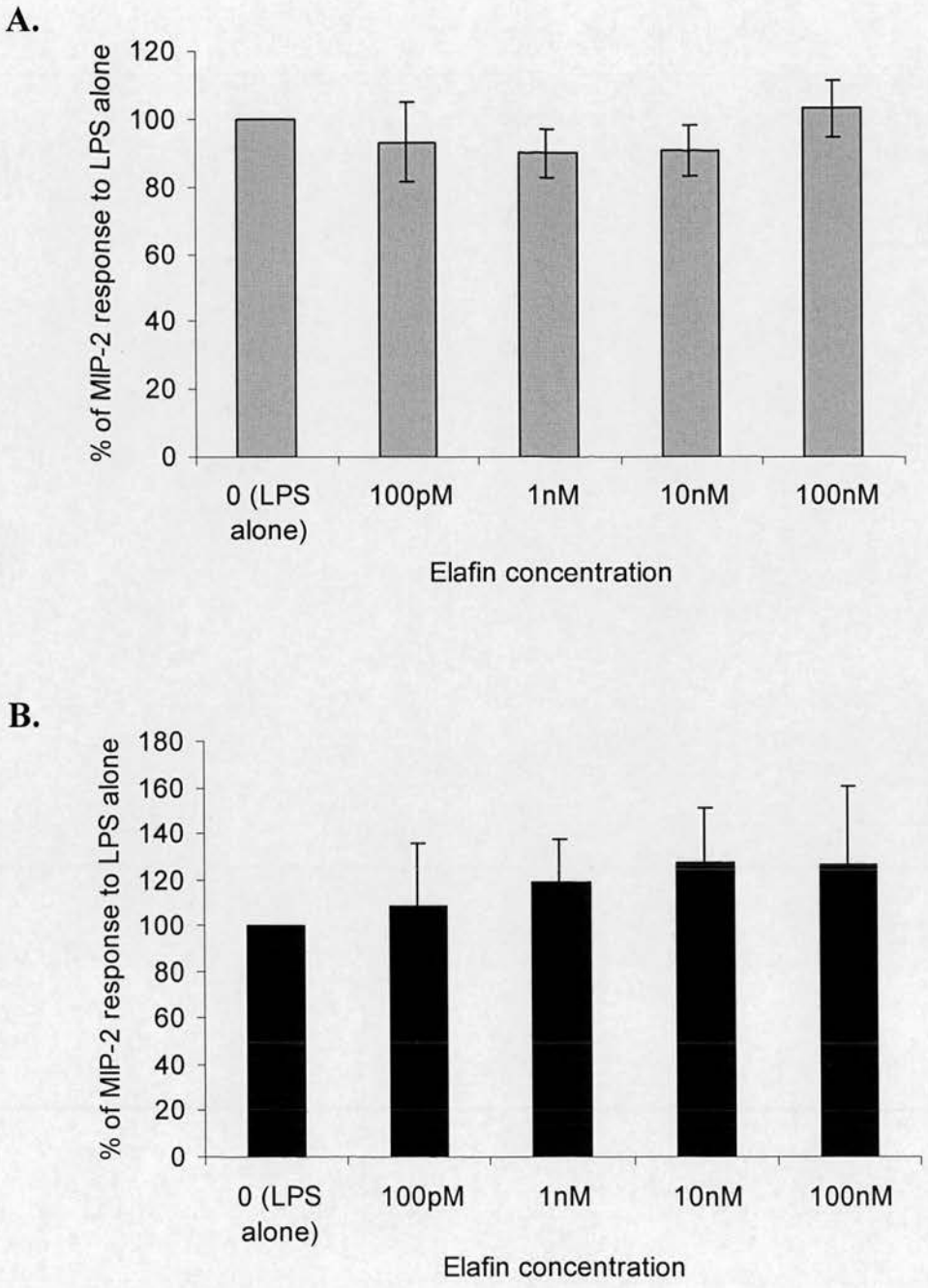


Figure 17. Full-length elafin has no significant effect on LPS-mediated MIP-2 secretion by murine Clara cells, in serum-free medium

Clara cells were seeded at 3 x 10^5 cells/well of a 48-well plate overnight, washed twice with PBS and 500µl serum-free DMEM added. 1µg/ml *E. coli* O55:B5 LPS was incubated with cells for 4 hours either alone or in the presence of increasing concentrations of elafin; media were analysed by MIP-2 ELISA. GRAPH A: Elafin and LPS added to cells simultaneously. GRAPH B: Elafin pre-incubated with LPS for 30 mins at 37°C prior to addition to cells. Values represent mean ± S.E. of n=4 (A) or n=5 (B) experiments, each performed in triplicate.

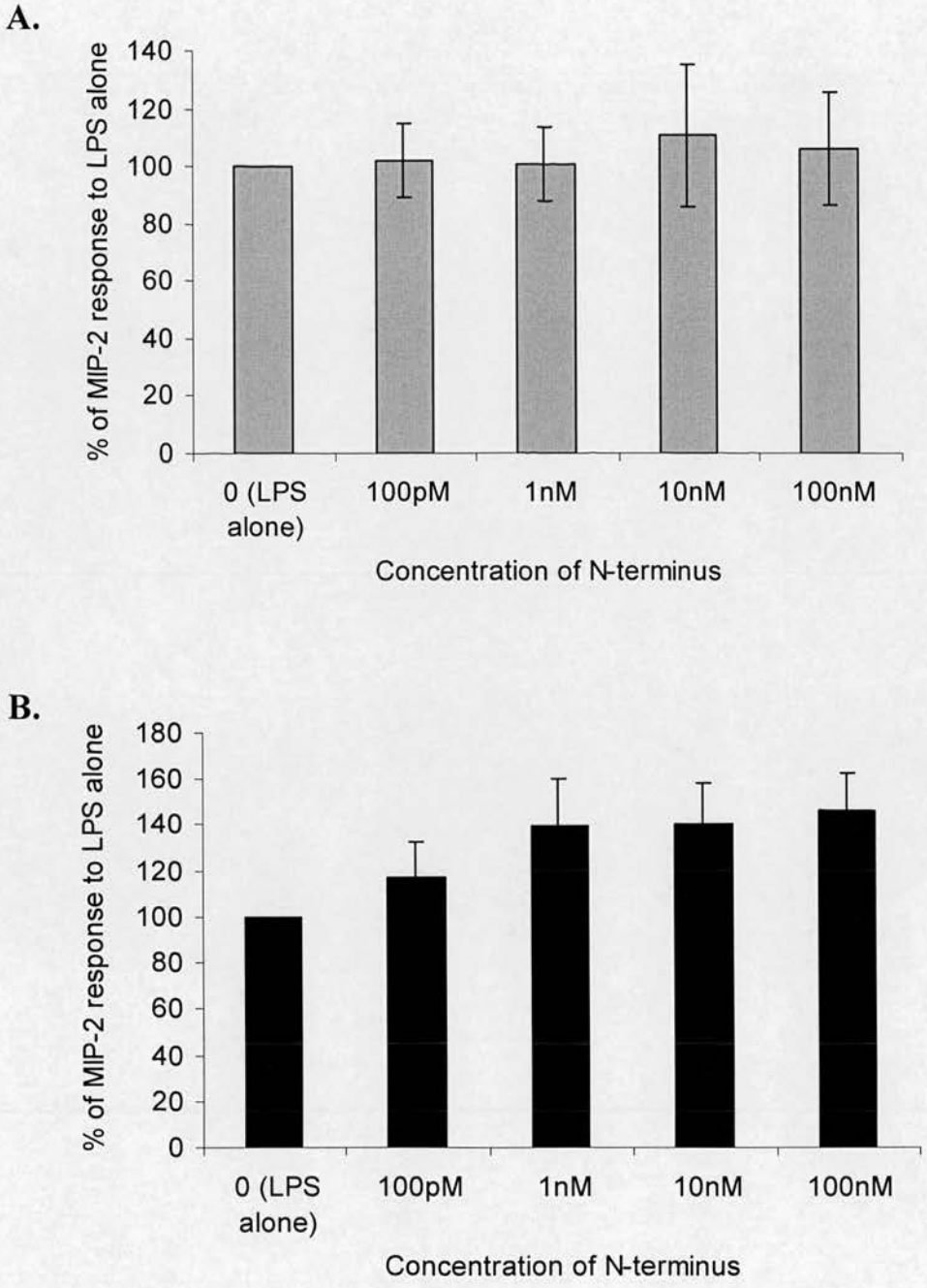


Figure 18. N-terminal elafin domain has no significant effect on LPS-mediated MIP-2 secretion by murine Clara cells, in serum-free medium

Clara cells were seeded at 3 x 10^5 cells/well of a 48-well plate overnight, washed twice with PBS and 500µl serum-free DMEM added. 1µg/ml *E. coli* O55:B5 LPS was incubated with cells for 4 hours either alone or in the presence of increasing concentrations of NH_2-terminus; media were analysed by MIP-2 ELISA. GRAPH A: NH_2-terminus and LPS added to cells simultaneously. GRAPH B: NH_2-terminus pre-incubated with LPS for 30 mins at 37°C prior to addition to cells. Values represent mean ± S.E. of n=5 experiments, each performed in triplicate.

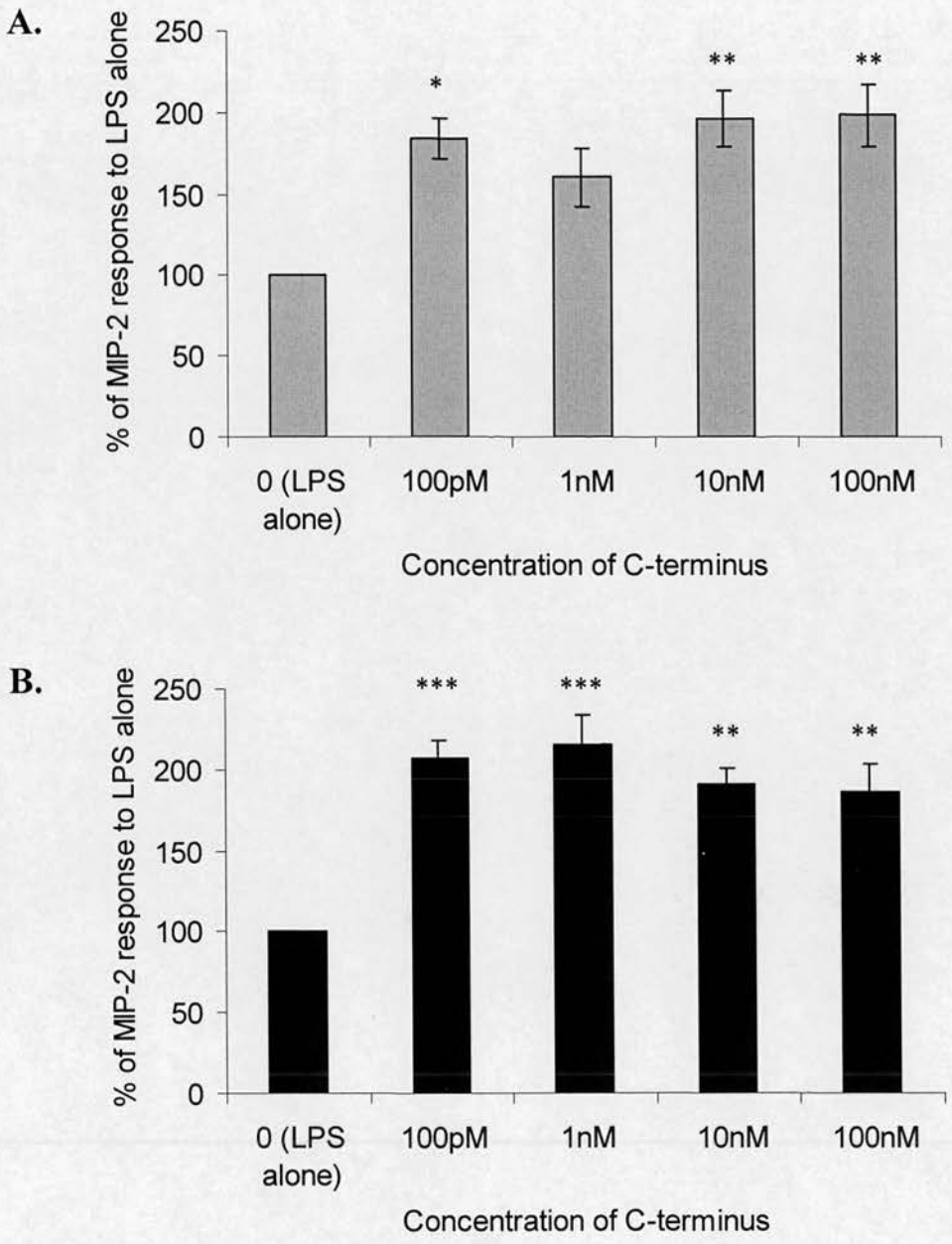


Figure 19. C-terminal elafin domain enhances LPS-mediated MIP-2 secretion by murine Clara cells, in serum-free medium

Clara cells were seeded at 3 x 10^5 cells/well of a 48-well plate overnight, washed twice with PBS and 500µl serum-free DMEM added. 1µg/ml *E. coli* O55:B5 LPS was incubated with cells for 4 hours either alone or in the presence of increasing concentrations of COOH-terminus; media were analysed by MIP-2 ELISA. GRAPH A - COOH-terminus and LPS added to cells simultaneously. GRAPH B – COOH-terminus pre-incubated with LPS for 30 mins at 37°C prior to addition to cells. Values represent mean ± S.E. of n=4 experiments, each performed in triplicate. * = significant difference, $P<0.05$, compared with 'LPS alone'. ** = significant difference, $P<0.01$, compared with 'LPS alone'. *** = significant difference, $P<0.001$, compared with 'LPS alone'.

4.3. DISCUSSION

The principal novel observation arising from the data in this chapter is that elafin is capable of directly modifying the inflammatory response of macrophages and epithelial cells to LPS. These findings suggest that the interactions of elafin with LPS, previously described in Chapter 3, may confer to elafin a physiological role in modulation of LPS-induced inflammation.

In initially selecting a cell type for use in these investigations, the macrophage was considered to be the ideal choice and for our studies we elected to use the RAW 264.7 murine macrophage cell line. This macrophage cell line has been used in a considerable number of studies as a model for macrophage activity; these studies include investigation of LPS-induced TNF-α secretion (Zhang *et al.*, 1999; Scott *et al.*, 2000b; Jarvis *et al.*, 2002), LPS-induced IL-1β secretion (Ndengele *et* al., 2000), LPS-induced IL-6 secretion (Scott *et al.*, 1999b), LPS-induced nitric oxide (NO) production (Kiemer *et al.*, 2002; Tsao *et al.*, 2002), LPS-mediated gene expression using gene array technology (Scott *et al.*, 2000a), binding and internalisation of LPS (Ding *et al.*, 1999; Kutuzova *et al.*, 2001), lipoteichoic acid (LTA)-induced TNF-α and IL-6 production (Scott *et al.*, 1999a), LTA-induced NO production (Kuo *et al.*, 2003) and direct killing of *Candida albicans* (Marcil *et al.*, 2002).

The role and importance of the macrophage in the inflammatory response has been discussed in depth in Chapter 1. As a major instigator of LPS-mediated effects *in vivo*, the macrophage can be considered a desirable target for modulating LPS-induced inflammation. Macrophages react to the presence of LPS in a hyper-responsive fashion and secrete a plethora of factors, including the classic pro-inflammatory cytokines IL-1, IL-6 and TNF-α (Dinarello, 1997); for the purpose of this investigation we used TNF-α as an inflammatory marker, since this cytokine generates many of the immunopathological features of LPS-induced shock (Beutler and Cerami, 1986). It is apparent that subverting the release of high concentrations of TNF-α into the circulation would be a valuable strategy in preventing LPS-mediated

tissue damage and sepsis. It was with this supposition in mind that these investigations were initiated.

However, since much of our interest lies in strategies to modulate inflammation in the lung, experiments were designed to model two different *in vivo* compartments: serum-containing conditions were investigated in order to model an environment such as the bloodstream, while serum-free conditions were used to model serum-deficient sites such as the airways.

In this regard, we also elected to model the responses of epithelial cells of the airways which, along with macrophages, may be the first line of defence against bacterial pathogens. Clara cells are nonciliated secretory cells which line the bronchiolar epithelium and play an important role in the repair of damaged airways (Boers *et al.*, 1999). These cells are known to comprise from 50% to 90% of cells lining the murine airways (Strayer *et al.*, 1998), and as such were considered an excellent model for epithelial cell activity. Much of the interest of studies into Clara cell biology to date has concerned their role in secretion of the anti-inflammatory protein Clara cell secretory protein, CC16 (also known as CC10 or CCSP). This molecule is a major component of the surface lining fluid of the lung and has been shown to play a role in modulating intrapulmonary inflammation, potentially by inhibiting the activity of phospholipase A_2 (Facchiano *et al.*, 1991; Johnston *et al.*, 1997; Hayashida *et al.*, 2000). Additionally, Clara cells respond to inflammatory stimuli such as TNF-α by releasing multiple inflammatory cytokines and chemokines, such as IFN-γ and MCP-1 (Park *et al.*, 2000). For our studies we chose to use MIP-2 as a marker of LPS-induced Clara cell activation since, unlike macrophages, epithelial cells are not major producers of TNF-α (Mannel and Echtenacher, 2000). MIP-2 is a C-X-C chemokine which, similar to its human homologue IL-8, is a potent mediator of neutrophil migration (Tekamp-Olson *et al.*, 1990).

While the results in this chapter were presented by taking each cell type in turn, discussion of these data will be approached by firstly examining the contrasting

observations in the presence or absence of serum, followed by a general discussion of potential mechanisms and implications for elafin functions *in vivo*.

4.3.1. Effects of elafin on LPS-mediated cellular activation in the presence of serum

Having established in Chapter 3 that binding of elafin could prevent interaction of LPS with LPS-binding protein, it seemed logical to extend these findings to an investigation of the effects of elafin on LPS-induced inflammation. Serum proteins such as LBP and sCD14 are known to play important roles in LPS-mediated cellular activation (Wright *et al.*, 1990; Hailman *et al.*, 1994; Tobias *et al.*, 1995; Hailman *et al.*, 1996). Therefore, prior to investigating the effects of elafin on LPS stimulation in serum-containing or serum-free conditions, it was deemed necessary to gain an understanding of the effects of serum in our system.

In these studies, macrophages responded to stimulation with LPS in a dose-dependent and serum-dependent manner (Figures 1-3). These observations, although important for establishing conditions for subsequent studies, are by no means novel (similar findings using the RAW 264.7 cell line have been reported by Lichtman *et al.*, 1998)); however it was of interest to note that the majority of the activity of serum in augmenting LPS activity appeared to be attributable to LBP (Figure 2). A study carried out by Wurfel *et al.* (1997) demonstrated that the blood of LBP knockout mice was profoundly hyporesponsive to LPS *ex vivo*, and that the LPS-induced response of leukocytes obtained from normal littermates was greatly suppressed when stimulation was performed in sera from LBP knockout mice. In spite of this, LBP knockout mice exhibited only slight hyporesponsiveness to LPS *in vivo*, suggesting that other molecules or mechanisms can mediate LPS-responsiveness in the host (Wurfel *et al.*, 1997). Our findings corroborate those of Wurfel *et al.* (1997) in suggesting the importance of serum LBP as a key mediator of LPS-induced cellular responses *in vitro*.

This rationale was of great significance for our investigations, since it was deemed desirable to minimise the presence of LBP in cellular assays, as a

consequence of the observation in Chapter 3 that LBP may outcompete elafin for binding to LPS. We wished to study the effects of elafin on LPS-mediated cellular stimulation using a test system in which elafin would not be overwhelmed, and therefore we chose to minimise the concentration of serum. Indeed, pilot experiments using 10% foetal calf serum serum in cell culture media suggested that elafin had little effect on LPS-mediated TNF-α secretion by macrophages (data not shown), and we hypothesised that this phenomenon was due to high LBP concentration. Thus, we elected to investigate the effects of elafin on LPS-mediated cellular stimulation in media containing 0.2% serum.

An essential pre-requisite to analysing elafin's effects was to determine whether elafin or its terminal domains themselves represent an inflammatory insult to cells. This was shown not to be the case using both cell types, in serum-containing or serum-free conditions (Figures 4, 8, 12 and 16). Recent studies have documented direct induction of inflammatory mediator release by cationic antimicrobial peptides; for example, a half-sized peptide of human SLPI containing the COOH-terminal domain induced TNF-α secretion by murine peritoneal macrophages (Sano *et al.*, 2003), while the human cathelicidin LL-37 directly induced production of the C-C chemokine MCP-1 by RAW 264.7 murine macrophages and the C-X-C chemokine IL-8 by A549 human epithelial cells (Scott *et al.*, 2002). However, it must be noted that these findings were obtained using peptides in the μM range, as compared with our studies which investigated the effects of elafin up to a maximum concentration of 100nM.

Most notably, we demonstrate here that elafin is capable of modulating LPS-mediated activation of both macrophages and Clara cells in serum-containing milieu. Strikingly, the effects of elafin were found to be divergent depending on the cell type, in that elafin dampened macrophage responses while enhancing those of Clara cells. Additionally, it was evident that pre-incubation of elafin with LPS prior to addition to cells was critical for facilitation of these effects.

In serum-containing milieu full-length elafin inhibited TNF-α secretion by macrophages by up to 40% (Figure 5B), whereas the same concentrations of elafin could enhance release of MIP-2 by Clara cells by more than 200% (Figure 13B) (both figures relative to the cellular response to LPS alone). Interestingly, the majority of the effects of the full-length elafin molecule appeared to be contributed by the COOH-terminal domain, since this was shown to be the more active domain using both cell types. In macrophage experiments, activity of full-length elafin was comparable with the COOH-terminus (Figure 5B and Figure 7B) while the NH_2-terminus did not significantly down-regulate TNF-α release (Figure 6B). These results support the findings in Chapter 3 which suggested that, although the NH_2-terminus appeared to bind LPS more strongly than the COOH-terminus, the COOH-terminus was the more effective portion in inhibiting LPS interaction with LBP (demonstrated by Figures 16 and 17 in Chapter 3); indeed, these Chapter 3 figures also demonstrated that the COOH-terminus may be more active than the full-length molecule. Similarly, while both elafin terminal domains up-regulated LPS-mediated MIP-2 secretion by Clara cells, the COOH-terminus was shown to be the more active domain (Figures 14 and 15). These findings suggest that the effects of the COOH-terminal domain may be modulated by the NH_2-terminus to a certain extent in the full-length elafin molecule; certainly, the termini do not appear to act synergistically in full-length elafin.

4.3.2. Effects of elafin on LPS-mediated cellular activation in the absence of serum

In stark contrast to the findings with macrophages in serum-containing media, pre-incubation of elafin peptides with LPS led to a greatly enhanced TNF-α response following macrophage stimulation in serum-free conditions. Full-length elafin augmented TNF-α secretion by as much as 300% (Figure 9B). The COOH-terminus was again found to be the more active domain, and in fact it was demonstrated to enhance LPS-induced TNF-α secretion even more effectively than full-length elafin (Figure 9B and Figure 11B).

These observations were corroborated by the studies carried out using Clara cells in serum-free conditions. Neither full-length elafin nor the NH_2-terminal domain significantly affected MIP-2 release (Figures 17 and 18), while the COOH-terminal domain greatly enhanced LPS-mediated activation, even in the absence of the pre-incubation step (Figure 19).

Taking into account the above findings which suggest that the elafin COOH-terminal domain is more active than the full-length molecule in enhancing cellular responses to LPS in the absence of serum, it is tempting to speculate that the elafin molecule may undergo proteolytic cleavage in tissues low in serum products (such as the airways) in order to release the more active domain. Of interest in this regard, elafin has also been isolated as a 6kDa (57 amino acid) peptide in psoriatic epidermis and bronchial secretions (Wiedow *et al.*, 1990; Sallenave and Silva, 1993); this form of elafin contains the entire COOH-terminal domain of the 9.9kDa elafin molecule (along with twelve NH_2-terminal residues), and may represent a proteolytic fragment with physiological activity.

Thus, in terms of the responsiveness of both macrophages and Clara cells to LPS in serum-free milieu, elafin's effects appear to be pro-inflammatory.

4.3.3. Potential mechanisms and functions for elafin *in vivo*

Hence it seems that exogenous elafin may play contrasting roles in modulation of LPS-mediated macrophage and epithelial cell activation, and these effects are further dependent on the presence or absence of serum during stimulation.

In sites rich in serum and serum products, such as the circulation, elafin may be beneficial in restricting overt secretion by macrophages of TNF-α and, potentially, other pro-inflammatory products. It is also a possibility that elafin may regulate secretion of anti-inflammatory cytokines, although time restraints prevented such studies within this investigation; such findings have previously been documented by Sano *et al.* (2000), who demonstrated that the homologous proteinase inhibitor SLPI could up-regulate production of IL-10 and transforming growth

factor-beta (TGF-β) by LPS-stimulated macrophages. Interestingly, researchers have failed to demonstrate any role for extracellular SLPI in down-regulating LPS-mediated TNF-α secretion by macrophages (Zhang *et al.*, 1997; Sano *et al.*, 2003). This observation could be explained by comparing the results obtained by these two groups with findings presented here. We demonstrated in Chapter 3 that SLPI can prevent the interaction of LPS with LBP, which is suggestive of a possible anti-inflammatory role for SLPI in LPS-induced macrophage activation. However, as with elafin these assays involved a pre-incubation step of peptide with LPS and this may also be important for SLPI; it is possible that SLPI may be out-competed for LPS-binding by LBP in serum. The study by Sano *et al.* (2003) used tissue culture conditions containing 10% serum (no mention was made of serum content in the paper by Zhang *et al.* (1997)), which as we found may be too rich in LBP to observe an effect (data not shown here). Moreover, both previous studies used high concentrations of LPS in the μg/ml range, compared with 50ng/ml used here, and thus the effects of SLPI may have been overwhelmed. Although the *E. coli* LPS serotype used in the Sano study was different to that used here, 10μg/ml of LPS may have presented too great an inflammatory insult. Finally, and perhaps critically, neither study involved a pre-incubation period of LPS with SLPI prior to addition to cells, a pre-requisite which we found to be essential in order to observe the effects of elafin.

A great many studies have suggested roles for cationic antimicrobial peptides in dampening macrophage responses to LPS, such as those describing activity of peptides derived from silk moth cecropin and bee melittin (Gough *et al.*, 1996; Scott *et al.*, 1999b), *Limulus* anti-LPS factor (LALF) and BPI (Dankesreiter *et al.*, 2000), polymyxin B (Coyne *et al.*, 1993), cathelicidins CAP18 and CAP11 (Nagaoka *et al.*, 2001), peptides derived from human lactoferrin (Zhang *et al.*, 1999) and the human defensins HNP-1 and HBD-2 (Scott *et al.*, 2000b).

Fewer studies have described a function for antimicrobial peptides in enhancing the release of pro-inflammatory mediators, however some findings merit discussion here. The study by Sano *et al.* (2003) demonstrated that a half-portion of

SLPI, representing the COOH-terminal domain of the holo-molecule, significantly up-regulated TNF-α secretion by LPS-stimulated macrophages. Although this study was performed in serum-containing conditions, their results may be suggestive of a similar activity for SLPI to that described here for elafin. Another study described by Bosshart *et al.* (2002) demonstrated that a number of arginine-rich cationic polypeptides such as hCAP37 and protamine can amplify LPS-induced IL-8 production by monocytes in human whole blood, and hypothesised that these peptides may directly facilitate transfer of LPS to CD14.

In our studies, elafin was shown to exert anti-inflammatory activity only when macrophages were stimulated with LPS in serum-containing conditions, and only when added to cells already in complex with LPS. It therefore appears that the interaction of elafin with LPS may prevent serum factors, such as LBP, from binding LPS and transferring it to CD14, thus dampening cellular responses. The same does not apply to activation of Clara cells, since even in serum-containing media elafin enhanced their response to LPS. Moreover, in the absence of serum elafin also acts to enhance LPS-mediated macrophage activation.

To aid understanding of potential mechanisms underlying these findings, it is necessary to examine differences in requirements for LPS activation in these two cell types. Epithelial cells are relatively hyporesponsive to stimulation with LPS, and this can perhaps be explained by findings concerning their expression of important molecules of the 'LPS receptor complex' (described in Chapter 1). While macrophages express surface TLR4 and mCD14, expression of these molecules by airway epithelial cells are more debatable. Whereas some studies have reported that TLR4 is not expressed on the surface of airway epithelial cells (Tsutsumi-Ishii and Nagaoka, 2003; Guillot *et al.*, 2004), a recent study has demonstrated membrane localisation of a low level of TLR4 in A549 (alveolar) epithelial cells, which was upregulated following viral stimulation (Monick *et al.*, 2003). The study by Guillot *et al.* (2004) also demonstrated that TLR4 resides intracellularly in human alveolar and bronchial epithelial cells, providing a rationale for prevention of chronic pulmonary inflammatory disease induced by continuous microbial exposure. Lack of surface

localisation in airway epithelial cells is not as a result of the absence of the accessory protein MD-2, since expression of this molecule has been demonstrated in human alveolar and tracheobronchial epithelial cells (Monick *et al.*, 2003; Guillot *et al.*, 2004).

While LPS-mediated activation of macrophages is known to occur via interactions with mCD14, knowledge surrounding a role for the membrane-bound form of CD14 in activation of epithelial cells is less well understood. A number of studies have suggested that pulmonary epithelial cells are mCD14-negative (Pugin *et al.*, 1993; Hedlund *et al.*, 2001), while others have demonstrated surface expression of CD14 (Becker *et al.*, 2000; Tsutsumi-Ishii and Nagaoka, 2003). However, the level of surface CD14 expression in these cells was very low compared with that of myeloid cells (Becker *et al.*, 2000). Recently it has been demonstrated that, while a tracheobronchial epithelial cell line expressed a low level of surface CD14, primary bronchial epithelial cells did not express surface CD14 (Guillot *et al.*, 2004). The latter finding is in agreement with a study by Striz *et al.* (1998) in which human bronchial epithelial cells were shown to be mCD14-negative; this study further suggested the importance of sCD14 in activation of these cells (Striz *et al.*, 1998). It therefore appears that the soluble form of CD14 may indeed be a key mediator of epithelial cell activation by LPS in the absence of mCD14, as suggested by various studies (Pugin *et al.*, 1993; Backhed *et al.*, 2002; Schulz *et al.*, 2002). Furthermore, sCD14 may also at least enhance the LPS-mediated activation of mCD14-bearing myeloid cells (Hailman *et al.*, 1996).

sCD14 is present in serum at concentrations of around 3-4μg/ml in healthy individuals, while this figure rises moderately but significantly in sepsis patients (Grunwald *et al.*, 1992; Landmann *et al.*, 1995). The 'catalytic' lipid transfer protein LBP, which transfers LPS monomers to CD14, is present in normal human serum at levels of approximately 5-15μg/ml (Calvano *et al.*, 1994; Gallay *et al.*, 1994; Froon *et al.*, 1995). However, during sepsis, acute phase serum levels of LBP can reach peak levels of up to 200μg/ml (Froon *et al.*, 1995). LBP and sCD14 are also present in lung fluids, although at much lower concentrations in the ng/ml range (Dubin *et*

al., 1996; Martin *et al.*, 1997). sCD14 may be released into the airways by alveolar macrophages (Hasday *et al.*, 1997), while recent findings have demonstrated that alveolar epithelial cells can produce and secrete LBP (Dentener *et al.*, 2000). It is also possible that airway epithelial cells may release sCD14, since intestinal epithelial cell lines have recently been shown to do so (Funda *et al.*, 2001).

It is apparent that excluding serum from culture media during LPS stimulation has a profound effect on the ability of LPS to induce inflammation, due primarily to the absence of both LBP and sCD14. Serum itself has also been shown to activate cells directly and induce their proliferation by activating signalling pathways. For example, serum has been shown to activate MAP kinases and can lead to phosphorylation of Raf and ERK2 (Cabedo *et al.*, 1996), and can also induce NF-κB activation via augmentation of the IκB kinases IKKα and IKKβ (Sasu and Beasley, 2000).

The observation in Chapter 3 that elafin can inhibit interaction of LPS with LBP (following direct binding to LPS) provides a straightforward rationale to help explain the inhibition of LPS-mediated macrophage activation in serum-containing milieu. It is equally possible that elafin could prevent interaction of LPS with other molecules of the LPS-receptor complex, such as CD14 or TLR4-MD2, either by binding to LPS or to these other key molecules. Elafin-mediated preclusion of any of these interactions could potentially dampen cellular activation. Appendix IV at the end of this chapter provides a diagrammatic representation of these proposed mechanisms, and also of those discussed below concerning macrophage and Clara cell activation in different serum conditions. The anti-inflammatory effects of elafin may also be mediated by an intracellular mechanism via abrogation of the signalling pathway downstream of TLR4. Morover, elafin could potentially interfere with intracellular LPS recognition by the NOD protein family, described in detail in Chapter 1 and recently reviewed by Inohara and Nunez (2003). In this regard, elafin may be internalised either alone (excess unbound elafin may be present in reaction mixtures added to the cells) or in complex with LPS, and inhibit a critical step prior to gene transcription. As yet an elafin receptor has not been identified, however

previous work has demonstrated that SLPI binds with high affinity to a receptor on the surface of monocytes and is internalised by these cells (McNeely *et al.*, 1997); this internalisation has been suggested by others as a critical step in the anti-inflammatory activity of exogenous SLPI (Mulligan *et al.*, 2000; Taggart *et al.*, 2002). It is additionally feasible that elafin-LPS complexes may be internalised following interaction with CD14 (Gegner *et al.*, 1995; Thieblemont and Wright, 1999; Vasselon *et al.*, 1999). It should be noted that potential roles for intracellular elafin in interfering with the LPS signalling pathway downstream of the receptor complex are discussed in depth in Chapter 5, and as such will not be subjected to great scrutiny here.

Although elafin-mediated inhibition of LPS interaction with molecules such as LBP may dampen macrophage activation in serum, the same was shown not to be true of Clara cell activation. Clara cell activation was enhanced by elafin in the presence of serum. As discussed above, it is likely that Clara cells are dependent on sCD14 to enhance their responsiveness to LPS due to absent or low level expression of mCD14. Moreover, Clara cell activation may rely upon sCD14-mediated LPS uptake and delivery to intracellular TLR4, as described in Chapter 1 (Thieblemont and Wright, 1999). It is possible that, in the case of Clara cells, elafin prevents binding of LPS by LBP as suggested previously, but that elafin functions to replace LBP in transfer of LPS from micelles to monomers. The elafin-LPS complex may potentially be recognised effectively by sCD14 (more-so than by membrane-bound CD14) and lead to augmented cellular activation. Equally, uptake and internalisation of elafin-LPS complexes or stimulation of a hitherto unidentified elafin receptor, proposed above as a mechanism of anti-inflammatory activity, could also potentially lead to enhancement of LPS-mediated activation.

Thus it may be hypothesised that, in defined circumstances, elafin may act as a 'carrier' molecule for LPS. In this model, elafin may function to replace serum factors to transfer LPS to the LPS recognition machinery.

In serum-free conditions elafin significantly up-regulated the LPS-mediated inflammatory response of macrophages; it is therefore suggested that elafin can play a catalytic role, similar to that of LBP, in transporting LPS monomers to the macrophage surface and, in particular, to mCD14. It is also feasible that elafin-LPS complexes may interact with TLR4-MD-2, or could be endocytosed as described above. In the presence of serum, however, elafin's net activity may be anti-inflammatory due to inhibition of the rapid LBP-mediated transfer of LPS to CD14.

Clara cell stimulation was enhanced slightly (but non-significantly) by full-length elafin and the NH_2-terminal domain in the absence of serum, while the COOH-terminal domain caused a significant increase in LPS-induced MIP-2 secretion. Similarly, this effect may be facilitated by elafin-mediated transfer of LPS to (potentially) mCD14 on the surface of Clara cells, by interaction of elafin-LPS with TLR4-MD-2, or by internalisation of LPS and recognition by intracellular TLR4 or NOD receptors. While in all other cases described thus far elafin only had effects following pre-incubation with LPS, in serum-free medium the COOH-terminus enhanced MIP-2 release even when added at the same time as LPS. The lack of requirement for prior interaction of COOH-terminus with LPS in this case is intriguing, and may be explained by the absence of sCD14 and LBP that would otherwise compete with the elafin peptide for binding to LPS in serum. Thus the COOH-terminus, which as discussed previously may exert even more potent activity than either full-length elafin or the NH_2-terminus, is unhindered in its interaction with LPS; as Clara cells may express only low levels (or have no expression at all) of surface CD14 and TLR4-MD-2, LPS activation of these cells is less efficient than macrophages, and thus the elafin peptide may have time to bind LPS prior to signalling.

To further delineate the findings of this chapter, the hypotheses proposed above could potentially be investigated using adenoviral overexpression techniques (such as those utilised in chapters 5 and 6 of this thesis) with which our laboratory has extensive experience. For example, selective augmentation of molecules of the LPS receptor complex in cells lacking these components and therefore unresponsive

to LPS, such as human embryonic kidney (HEK) 293T cells (see Medvedev and Vogel, 2003), could aid understanding of how elafin could act as an LPS 'carrier' protein in serum-free conditions. Adenovirus-mediated overexpression of TLR4-MD-2 in these cells may provide an answer as to whether elafin can deliver LPS directly to this complex in the absence of LBP or CD14. Moreover, overexpression of mCD14 could potentially reveal a role for elafin in mediating LPS transfer to this molecule, prior to recognition by TLR4-MD-2. Similar studies could be also used to augment NOD proteins such as NOD1 in HEK 293T cells (shown previously by Inohara *et al.* (2001) to confer LPS-responsiveness) in order to investigate whether elafin-LPS complexes can be internalised and delivered to these intracellular receptors. Such overexpression studies could equally be performed in the cell types used in this study, however initially it would perhaps be more informative to make use of a naïve cell type with an LPS-unresponsive phenotype.

With regards to macrophage activation in serum-containing milieu, selective monoclonal antibody-mediated blocking of LBP would perhaps help to address the question of whether this is the key molecule in conversion of elafin from an anti-inflammatory agent to a pro-inflammatory one: in the presence of LBP in serum, elafin may prevent LBP-LPS interaction and provide an anti-inflammatory phenotype, whereas in the absence of LBP's catalytic activity elafin may be free to bind LPS and itself act as a carrier molecule. Analogous studies to block sCD14 could be performed in the case of Clara cells to test the previous hypothesis that elafin can enhance LPS-mediated activation by transferring LPS to sCD14 in serum.

Regardless, initial findings presented here suggest that elafin may play a role in down-regulating potentially deleterious systemic responses to endotoxin, mediated by TNF-α release by activated macrophages; conversely, elafin may enhance local immune responses to LPS or to bacterial pathogens in serum-deficient sites by augmenting the inflammatory response.

Elafin may thus play a pro-inflammatory role in the control of local responses to Gram-negative bacterial infection in the airways. These findings may be

particularly relevant in the human lung since elafin secretion may be associated with alveolar epithelium, Clara cells, or tracheal epithelium (Sallenave *et al.*, 1993; Nara *et al.*, 1994), and is augmented by pro-inflammatory mediators such as IL-1, TNF, HNE and LPS (Sallenave *et al.*, 1994; Reid *et al.*, 1999).

It may be of benefit to the host to enhance migration of neutrophils and other myeloid cells into the lung epithelium in order to combat bacterial invasion and aid in the clearance of pathogens. However, an over-zealous immune response may lead to interstitial tissue damage; in this regard, elafin may play an important role in enhancing inflammatory responses locally while preventing the potentially detrimental effects of exogenous proteases. Interestingly, another potentially pro-inflammatory role for elafin may be suggested by studies which have demonstrated that proteinase-3 (PR-3) and HNE can degrade CD14 on the surface of monocytes, leading to dampening of TNF-α production (Le-Barillec *et al.*, 1999; Yard *et al.*, 2002). In inflamed tissues characterised by infiltration of monocytes/macrophages and neutrophils, elafin may inhibit this proteinase-mediated CD14 degradation and thus perpetuate the inflammatory response. A similar role could be proposed for SLPI, which also inhibits the activity of another CD14-degrading proteinase, cathepsin G (Le-Barillec *et al.*, 2000).

The concentrations of elafin investigated here could well be relevant to the *in vivo* situation. For example, elafin levels in the serum of healthy humans have been measured at around 8-15ng/ml (approximately 1nM), while patients with psoriasis may have serum elafin levels as high as 300ng/ml (approximately 30nM) (Alkemade *et al.*, 1995); the majority of elafin's effects observed here were induced at around 10-100nM. While elafin levels have not been measured in serum during human sepsis, levels of SLPI have been shown to rise from approximately 43ng/ml (3.7nM) to 150ng/ml (12.8nM) in septic surgical patients (Grobmyer *et al.*, 2000). Moreover, plasma concentrations of SLPI have been documented to reach 176ng/ml (15nM) in patients with pneumonia (Duits *et al.*, 2003b).

With regards to elafin levels in the airways, these may also be within or exceeding the range tested in the studies described here. In bronchoalveolar lavage (BAL) fluid from normal subjects, elafin has been detected at concentrations around 3nM (Tremblay *et al.*, 1996); this figure was found to be elevated to around 120nM in BAL fluid from patients with active farmer's lung, a common form of hypersensitivity pneumonitis (induced by inhalation of organic dust from hay) which is characterised by influx of alveolar neutrophils and macrophages (Tremblay *et al.*, 1996). Similarly, levels of elafin in BAL fluid were shown to rise from less than 5nM in healthy individuals to as much as 325nM in patients with established acute respiratory distress syndrome (ARDS) (Sallenave *et al.*, 1999a). The level of elafin in epithelial lining fluid has not been determined, but is likely to be several orders of magnitude greater than that measured in BAL fluid.

The findings of this chapter additionally corroborate recent studies regarding activity of elafin in murine models. Simpson *et al.* (2001a) demonstrated that adenovirus-mediated overexpression of elafin in murine airways augmented TNF-α and MIP-2 levels in BAL fluid following LPS administration, and these increases were associated with enhanced LPS-induced airway neutrophilia (Simpson *et al.*, 2001a). In good agreement with the concentrations of elafin tested here, Simpson *et al.* (2001a) measured adenovirus-derived (since no murine elafin homologue exists) elafin levels in BAL fluid of up to around 140ng/ml (14nM). Furthermore, work carried out using transgenic mice expressing human elafin has demonstrated that these mice exhibit lower serum-to-BALF ratios of pro-inflammatory cytokines (TNF-α, MIP-2 and MCP-1) than wild-type mice, following LPS administration to the lungs; these cytokine levels were correlated with elafin concentrations (pg/ml range) in BAL fluid, and were also associated with an increase in neutrophil and macrophage influx (Sallenave *et al.*, 2003). Conversely, in response to systemic LPS, mice expressing elafin had lower levels of serum TNF-α than wild-type mice, while peritoneal macrophages from elafin mice were hypo-responsive to LPS (Sallenave *et al.*, 2003). The data described in this chapter may help to further clarify these *in vivo* observations.

4.4. SUMMARY

The results presented in this chapter demonstrate that the LPS-binding properties of elafin, described in Chapter 3, confer to the elafin molecule the ability to modulate the pro-inflammatory activity of LPS *in vitro*. Serum conditions were shown to be an important determinant of the effects of elafin and these findings may be of relevance to potential roles for elafin *in vivo*.

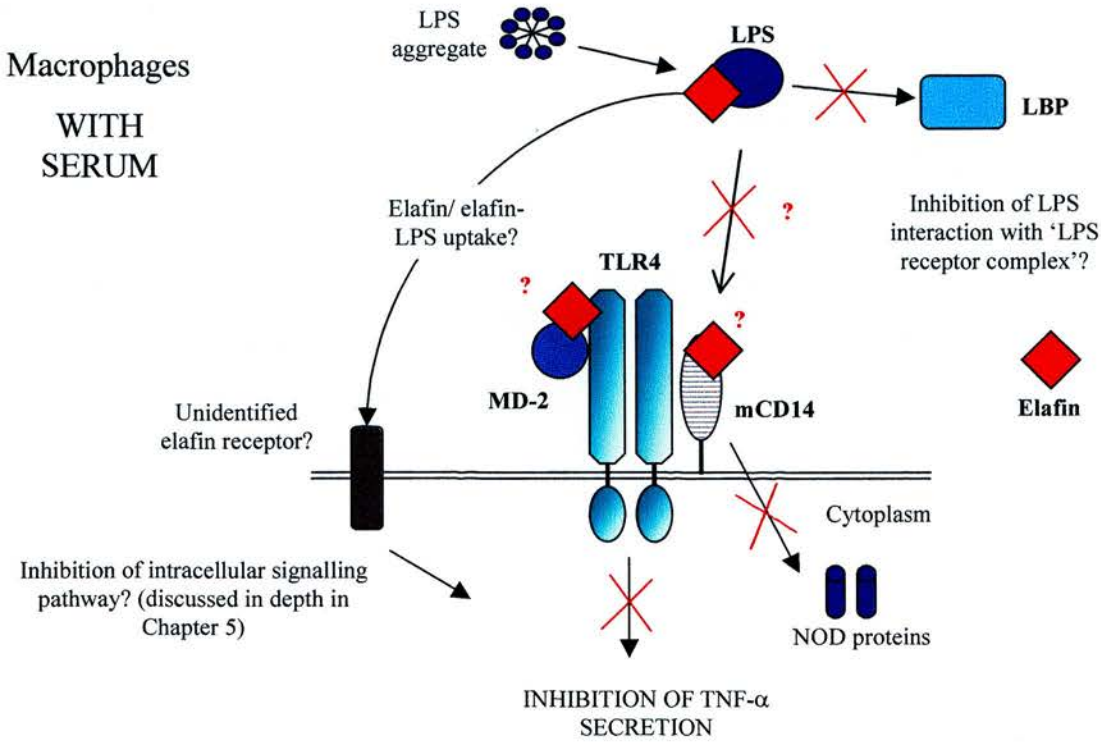
Elafin may play a role in innate immunity by modulating host responses to Gram-negative bacterial LPS: elafin could function to enhance immune responses in sites of local inflammation, such as the airways, but to down-regulate potentially deleterious responses to LPS in the circulation.

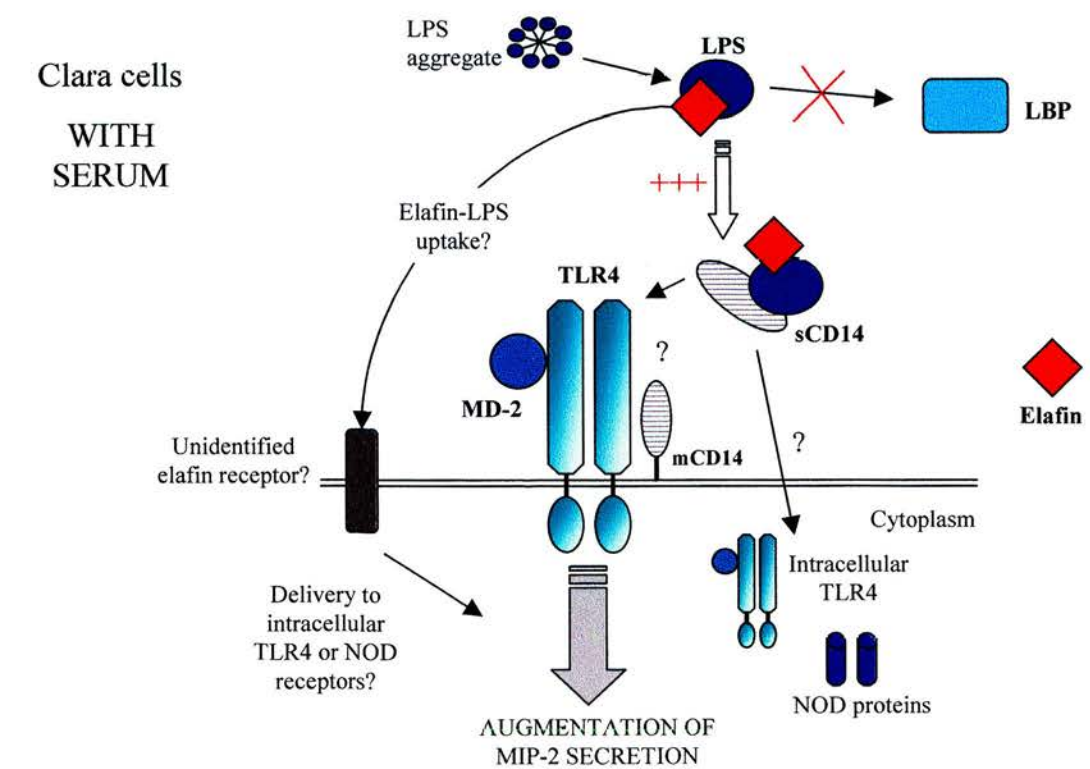
APPENDIX IV

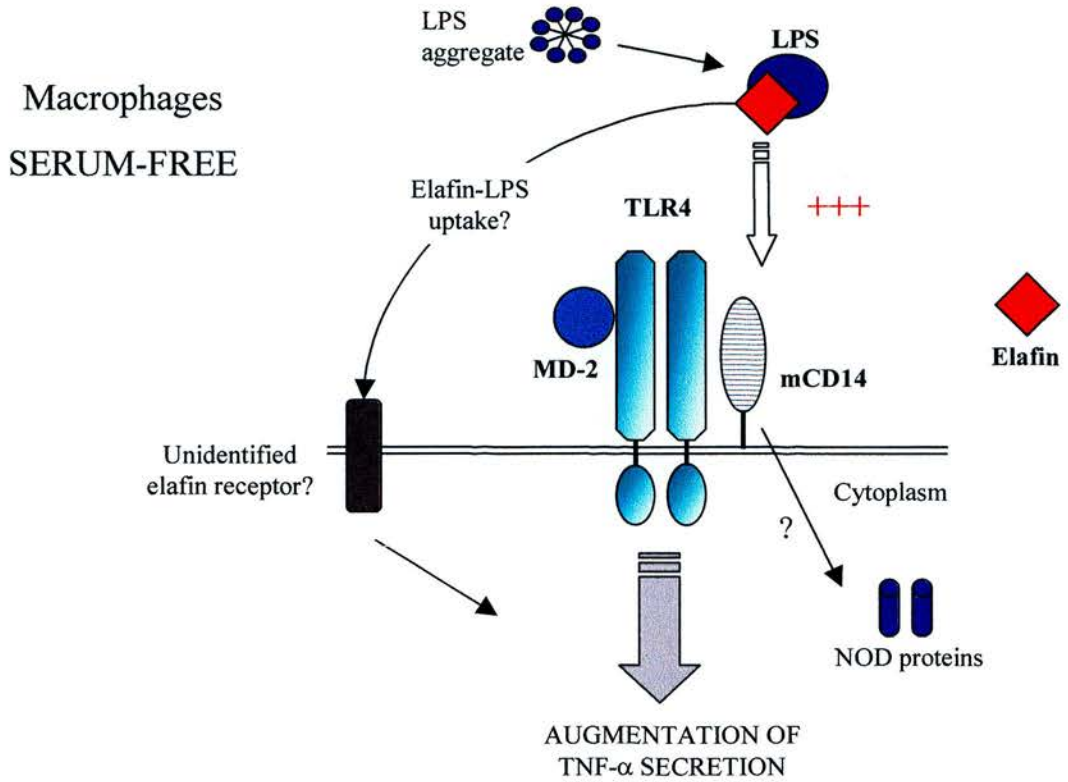
Schematic diagram of potential mechanisms of the effects of elafin on LPS-mediated activation of macrophages and Clara cells

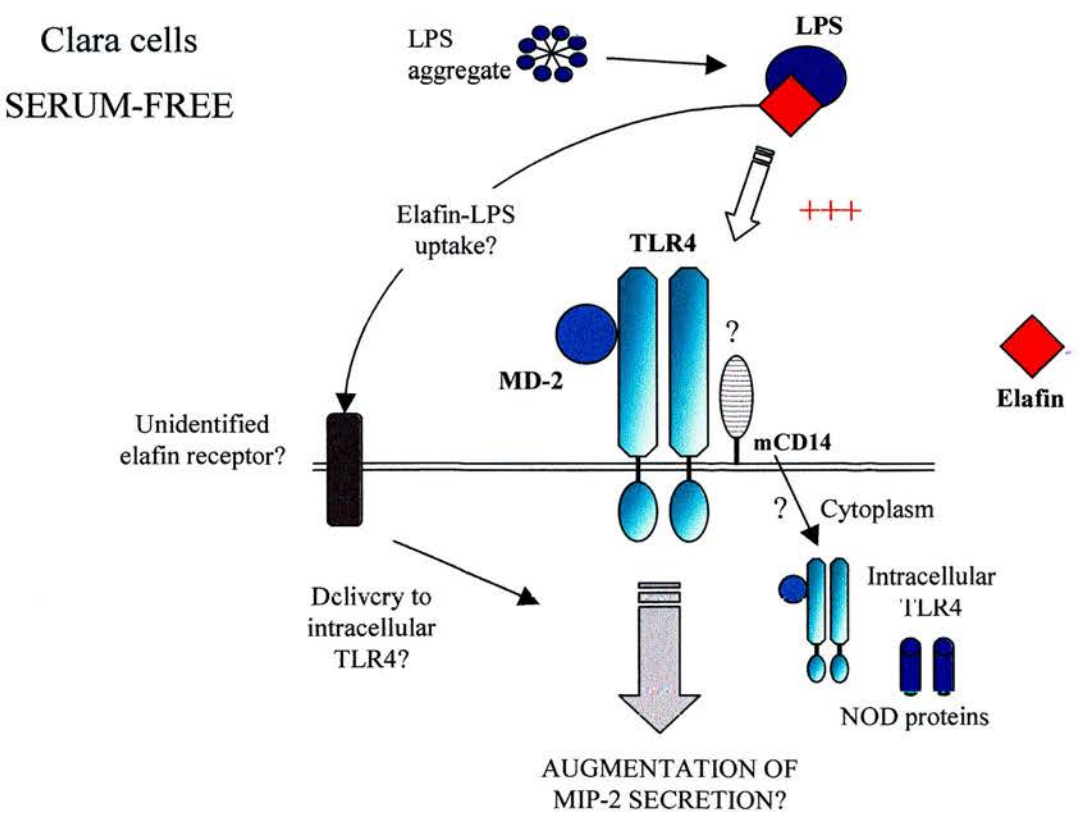
Potential mechanisms to explain the elafin-mediated effects in this chapter are depicted diagrammatically. Elafin is represented by a red diamond to indicate possible interactions with the receptor complex. Question marks denote points of particular uncertainty. 'X' across an arrow denotes inhibition of a step in the pathway; '+++' indicates augmentation.

For description of the potential mechanisms represented in these diagrams, please refer to section 4.3.3. under 'Discussion' in Chapter 4.


Macrophages
WITH
SERUM
LPS
aggregate
LPS
LBP
Elafin/ elafin-
LPS uptake?
?
Inhibition of LPS
interaction with 'LPS
receptor complex'?
TLR4
?
?
MD-2
mCD14
Elafin
Unidentified
elafin receptor?
Cytoplasm
Inhibition of intracellular signalling
pathway? (discussed in depth in
Chapter 5)
NOD proteins
INHIBITION OF TNF-α
SECRETION


Clara cells
WITH
SERUM
LPS
aggregate
LPS
LBP
+++
Elafin-LPS
uptake?
TLR4
sCD14
?
MD-2
Elafin
Unidentified
elafin receptor?
mCD14
?
Cytoplasm
Intracellular
TLR4
Delivery to
intracellular
TLR4 or NOD
receptors?
NOD proteins
AUGMENTATION OF
MIP-2 SECRETION


Macrophages
SERUM-FREE
LPS aggregate
LPS
Elafin-LPS uptake?
TLR4
+++
MD-2
mCD14
Elafin
Unidentified elafin receptor?
Cytoplasm
?
NOD proteins
AUGMENTATION OF TNF-α SECRETION


Clara cells
SERUM-FREE
LPS aggregate
LPS
Elafin-LPS uptake?
TLR4
+++
?
MD-2
Elafin
Unidentified elafin receptor?
mCD14
?
Cytoplasm
Delivery to intracellular TLR4?
Intracellular TLR4
NOD proteins
AUGMENTATION OF MIP-2 SECRETION?

CHAPTER 5

THE EFFECT OF ELAFIN GENE AUGMENTATION ON THE INFLAMMATORY RESPONSE OF MACROPHAGES TO LPS *IN VITRO*

5.1. AIMS

The principal aim of the work described in this chapter was to study the effects of elafin gene augmentation on the inflammatory response of murine macrophages to LPS, using an adenovirus-based strategy. This gene transfer approach uses a replication-deficient adenovirus vector (Ad), encoding human elafin cDNA under the control of the powerful murine cytomegalovirus (MCMV) promoter, to facilitate cellular overexpression of elafin (Sallenave *et al.*, 1998). Simpson *et al.* (2001b) have previously used this technique to demonstrate that adenoviral augmentation of elafin protects human alveolar epithelial (A549) cells against the injurious effects of both HNE and whole activated neutrophils *in vitro*, and murine lungs against acute inflammatory injury mediated by *P. aeruginosa in vivo*. Moreover, adenoviral overexpression of elafin has been shown to enhance human neutrophil migration *in vitro*, and to augment LPS-mediated neutrophil migration into murine airways *in vivo* (Simpson *et al.*, 2001a).

The experiments described herein were undertaken to investigate whether similar genetic augmentation of elafin could modulate the vigorous macrophage response to LPS *in vitro*. Chapter 4 demonstrated that exogenous elafin peptides can directly modulate the LPS-mediated inflammatory response of macrophages, and that this activity can be pro- or anti-inflammatory depending on the absence or presence of serum, respectively. While the use of synthetic peptides facilitated investigation of elafin's extracellular effects on LPS activation, it was envisaged that genetic overexpression could potentially delineate any intracellular effects of elafin. It must be noted that, due to time constraints, the following experiments were carried out using only cell culture conditions incorporating serum. Thus, the experiments

described in this chapter set out to model the effects of Ad-elafin on macrophage responses to LPS in serum-containing sites, such as the circulation.

5.2. RESULTS

5.2.1. Experiments to establish conditions for optimal Ad-elafin infection of RAW 264.7 murine macrophages

Prior to commencing investigation of the effects of elafin overexpression on LPS-mediated activation of macrophages, it was deemed necessary to establish a set of optimal conditions for adenoviral infection of these cells. It is known that infection of macrophages with adenovirus vectors is feasible using a high multiplicity of infection (moi), but is relatively inefficient due to a low level of cellular expression of the Coxsackie/adenovirus receptor (CAR) (Kaner *et al.*, 1999; Worgall *et al.*, 1999). For example, Kaner *et al.* (1999) demonstrated that alveolar macrophages expressed Ad vector transgenes 100 to 1000-fold less efficiently than A549 cells. However, adenoviral gene transfer to cells lacking the receptor activity to bind adenovirus fibre protein has been shown to be enhanced by generation of calcium phosphate ($CaPO_4$ or CaPi) precipitates in the infection milieu (Fasbender *et al.*, 1998); such precipitates have also been used previously to aid transfection of plasmid DNA into cultured cell lines (Chen and Okayama, 1988). Using this technique, Ad:CaPi co-precipitates can markedly augment transgene expression by increasing fibre-independent binding of virus to cells (Fasbender *et al.*, 1998).

On consideration of these findings, it seemed prudent to initially investigate the efficiency of adenoviral infection of RAW 264.7 murine macrophages, and the effects of incorporating CaPi precipitates into viral infection media. For this purpose, comparisons were made between efficiency of gene transfer when infection was performed either in the DMEM used to maintain cells on a day-to-day basis, or in media prepared to contain CaPi precipitates. In the latter case, calcium chloride ($CaCl_2$) was added to a Minimum Essential Eagle's Medium (MEEM) formulation containing 1.8mM Ca^{2+} and 0.86mM Pi, thus forming an infection medium containing microscopically apparent CaPi precipitates.

Figure 1 demonstrates that infection of RAW 264.7 cells with the Ad-lacZ reporter construct could be achieved when infection was performed in DMEM, with infection efficiency around 30% at moi 100 (Panel B). Incubating Ad-lacZ with cells in MEEM without addition of Ca^{2+}, and therefore without CaPi co-precipitates, resulted in infection efficiency around 50% (Panel D). However, incorporation of 4mM Ca^{2+} into MEEM resulted in formation of CaPi precipitates in the viral infection media, with a concomitant increase in infection efficiency to approximately 100% (Panel F); in this case, precipitates were formed with a final concentration of 5.8mM Ca^{2+} and 0.86mM Pi. Incubation of macrophages with CaPi precipitates did not appear to effect any morphological damage to the cells, and this observation was supported by performing cell counts and trypan blue exclusion (data not shown).

In order to investigate efficiency of infection of cells with Ad-elafin, cells were infected with the viral construct in a range of infection media and at varying moi, and cell supernatants and lysates were analysed by elafin ELISA (Figures 2 and 3). As suggested by Figure 1, elafin production was greatly enhanced by incorporation of CaPi precipitates into infection media, and more specifically this efficiency could be augmented by increasing the concentration of Ca^{2+} added to MEEM to form precipitates (Figure 2). Relatively little elafin was produced by Ad-elafin-infected cells in DMEM or MEEM without addition of Ca^{2+}, and elafin production was optimal when 4mM Ca^{2+} was added. Both secreted and intracellular elafin levels were measured; the observation that similar dose responses were obtained with secreted and intracellular elafin supports findings using trypan blue exclusion (not shown) that cells were intact following incubation with Ad:Pi, and suggests that extracellular elafin levels were not associated with cell lysis (Figure 2). Figure 2 demonstrates data pertaining to an Ad-elafin moi of 100, but the level of elafin production could also be affected by altering the moi while using a constant final concentration of 5.8mM Ca^{2+}/0.86mM Pi in infection media (Figure 3). Notably, uninfected cells produced no detectable elafin since no murine homologue of elafin has been identified to date, and the anti-elafin antibody did not cross-react with any cell-derived protein. Similar dose-response relationships were again obtained for extra- and intracellular elafin levels (Figure 3).

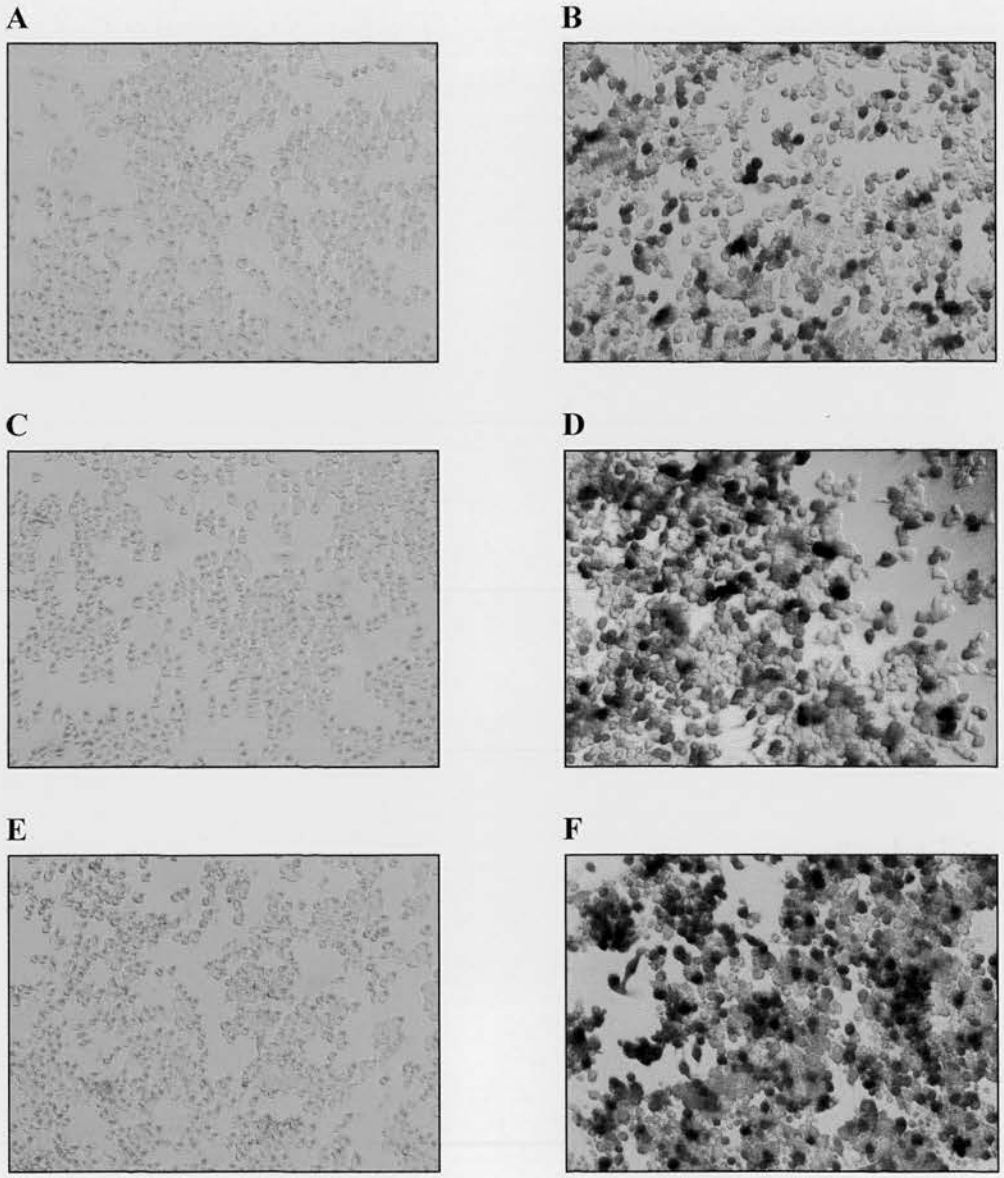


Figure 1. Calcium phosphate (CaPi) precipitates enhance the efficiency of adenoviral infection of RAW 264.7 murine macrophages

RAW 264.7 murine macrophages (5 x 10^5/well of a 48-well plate) were treated with vehicle alone or Ad-lacZ at moi 100, and incubated in DMEM containing 0.2% serum for 18-20 hours at 37°C. Cells were then subjected to X-gal staining as described in 'Materials and Methods'. Blue colouration indicates expression of the β-galactosidase gene. DMEM=Dulbecco's Modified Eagle's Medium; MEEM = Minimum Essential Eagle's Medium.

PANEL A - no viral infection of cells, incubated in DMEM
PANEL B - cells infected with Ad-lacZ (moi 100) in DMEM
PANEL C - no viral infection of cells, incubated in MEEM
PANEL D - cells infected with Ad-lacZ (moi 100) in MEEM
PANEL E - no viral infection of cells, incubated in MEEM plus 4mM Ca^{2+}
PANEL F - cells infected with Ad-lacZ (moi 100) in MEEM plus 4mM Ca^{2+}

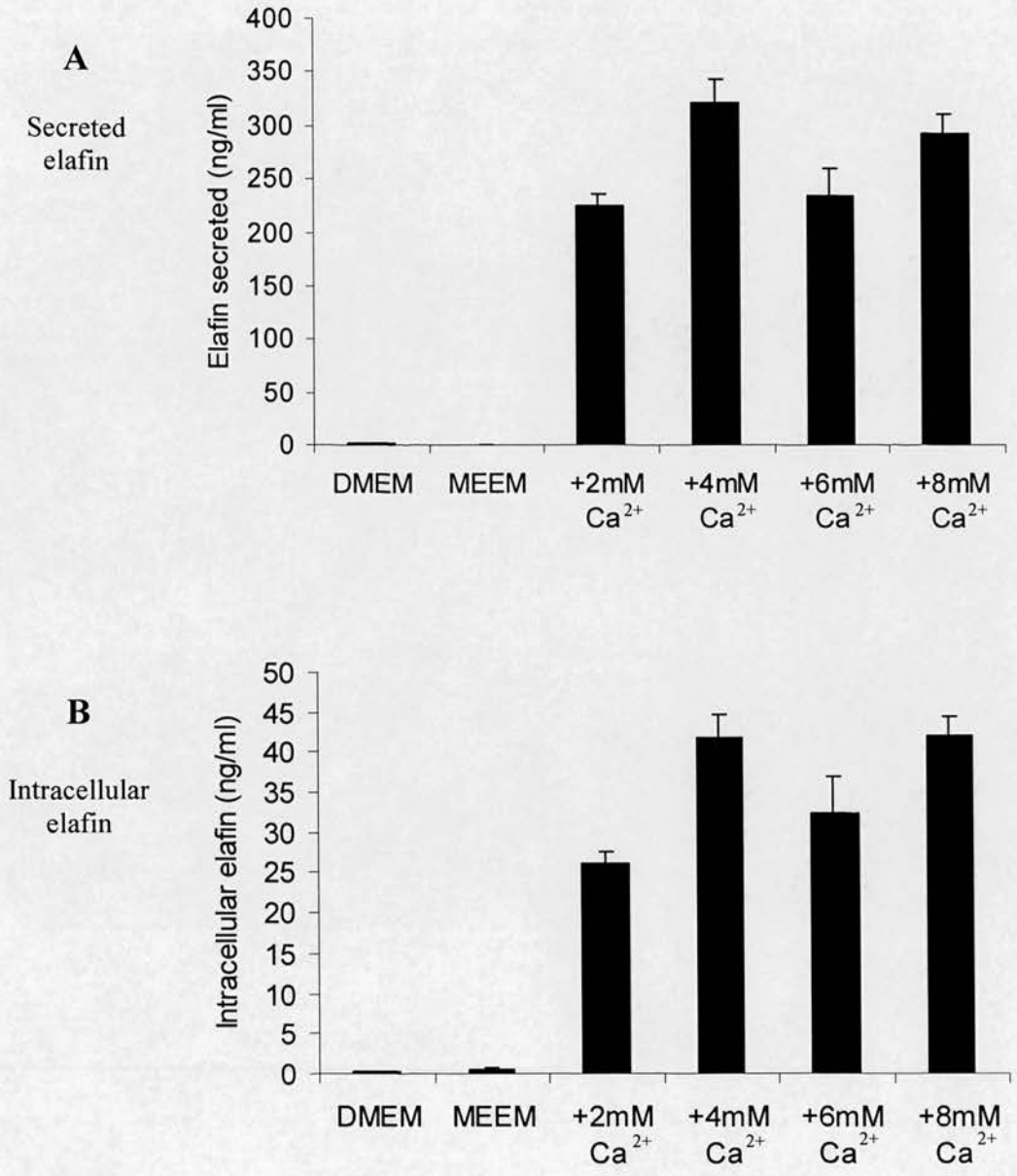


Figure 2. Increasing the concentration of Ca^{2+} in infection medium augments elafin production by Ad-elafin-infected RAW 264.7 macrophages

RAW 264.7 murine macrophages were seeded at 5 x 10^5 cells/well of a 48-well plate overnight. Cells were then infected with Ad-elafin (moi 100) for 45 minutes at 37°C in 250µl DMEM or in 250µl MEEM with varying concentrations of Ca^{2+} added. Cells were washed twice with PBS, 500µl DMEM containing 0.2% FCS added and cells incubated for 23 hours. Media were analysed by elafin ELISA to measure levels of secreted elafin (A). Alternatively, cells were lysed to obtain total protein and lysates analysed by ELISA for intracellular elafin (B). Values represent mean ± S.E. of n=3 experiments, each performed in triplicate.

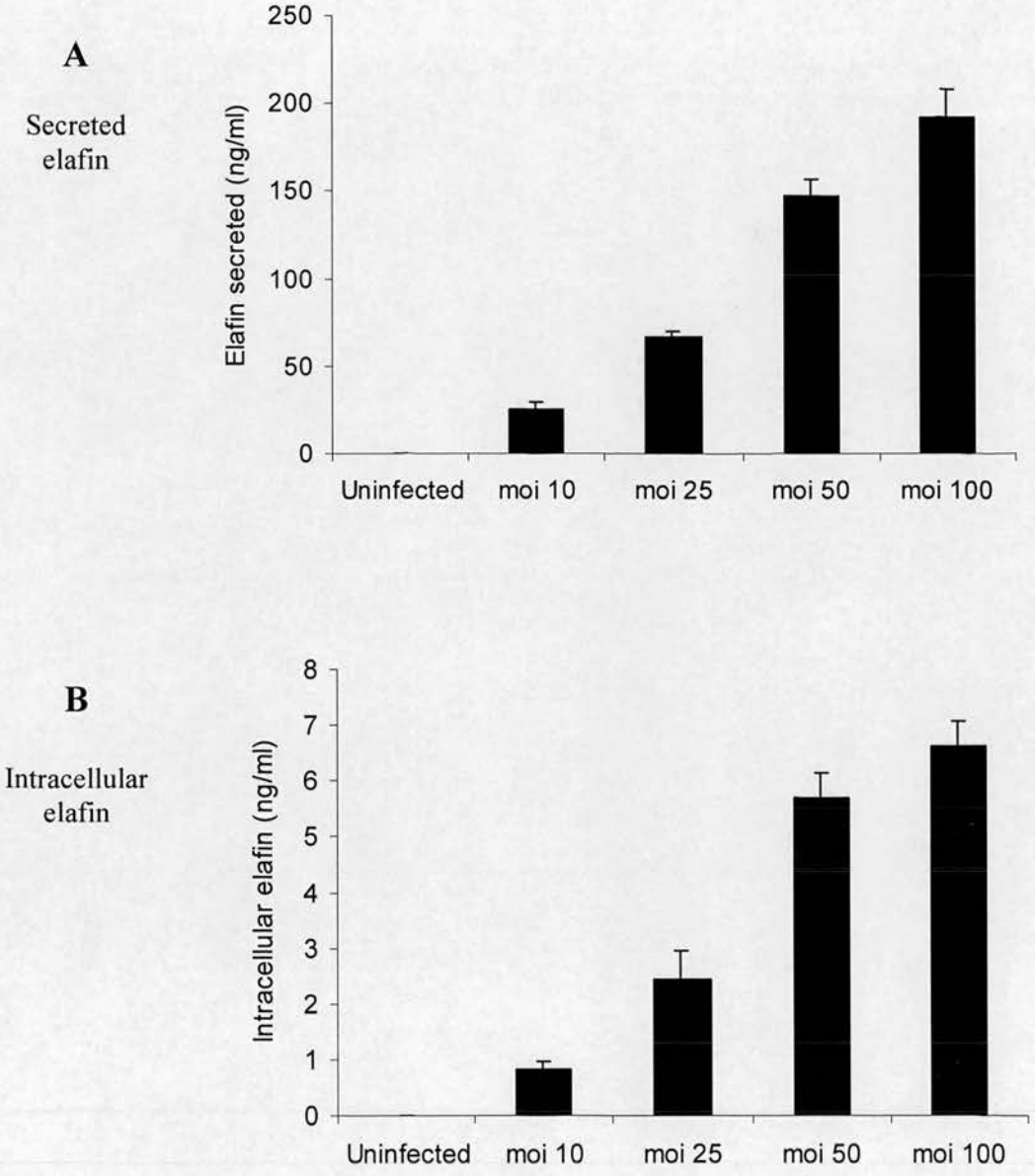


Figure 3. Increasing the multiplicity of infection (moi) of Ad-elafin augments elafin production by RAW 264.7 macrophages

RAW 264.7 murine macrophages were seeded at 5 x 10^5 cells/well of a 48-well plate overnight. Cells were then infected with Ad-elafin at increasing moi for 45 minutes at 37°C, in 250µl MEEM containing 5.8mM Ca^{2+}/0.86mM Pi. Cells were washed twice with PBS, 500µl DMEM containing 0.2% FCS added and cells incubated for 23 hours. Media were analysed by elafin ELISA to measure levels of secreted elafin (A). Alternatively, cells were lysed to obtain total protein and lysates analysed by ELISA for intracellular elafin (B). Values represent mean ± S.E. of n=3 experiments, each performed in triplicate.

Within the range of mois tested, elafin production was maximal at moi 100; moreover, cell viability was unaffected at this moi. On consideration of the results shown in Figures 2 and 3, all subsequent viral infections were performed using an moi of 100, and with infection media comprising MEEM with 4mM Ca^{2+} added (giving a final concentration of 5.8mM Ca^{2+}/0.86mM Pi to form precipitates).

Additionally, it was deemed necessary to investigate the effects of CaPi precipitates on the TNF-α response of cells since, although this treatment did not appear to directly affect cell viability, it was important that cells would retain their LPS-responsive activity for subsequent assays. CaPi precipitates were demonstrated to be a considerable inflammatory stimulus to macrophages; over a 23-hour period, cells which had been incubated for 45 minutes with CaPi secreted a significantly greater concentration of TNF-α than cells which had been incubated for 45 minutes with DMEM (Figure 4). It should be noted that 45 minutes represents the incubation period used for adenoviral infections, and cells would normally be stimulated with LPS 23 hours post-infection. However, despite these observations, incubation of cells with CaPi had no significant effects on their subsequent inflammatory response to LPS stimulation, as compared with cells incubated with DMEM (Figure 5). It was therefore concluded that the use of CaPi precipitates to enhance elafin gene augmentation was a practicable strategy for our investigations.

5.2.2. The effect of LPS on elafin production by Ad-elafin-infected RAW 264.7 murine macrophages

Previous work has demonstrated that LPS can upregulate elafin production by Ad-elafin-infected human A549 cells and human monocyte-derived macrophages *in vitro*, and also elafin secretion in murine airways treated with Ad-elafin (Simpson *et al.*, 2001a). This effect has been attributed to the presence of LPS-responsive elements within the MCMV promoter (Loser *et al.*, 1998; Simpson *et al.*, 2001a). Assays were performed to investigate whether LPS has similar effects on elafin production by Ad-elafin-infected murine macrophages in this system.

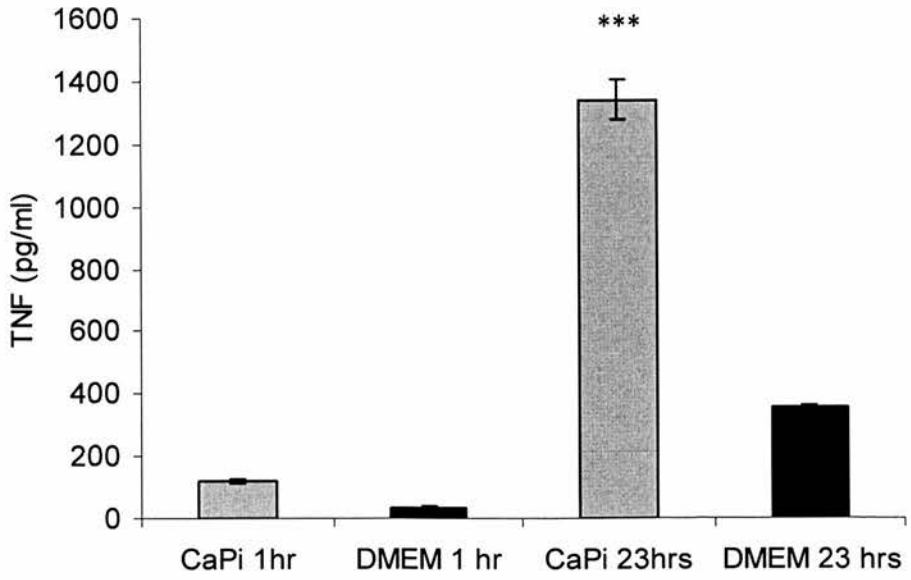


Figure 4. Calcium phosphate (CaPi) induces a more pronounced inflammatory response from RAW 264.7 macrophages than DMEM

RAW 264.7 murine macrophages were seeded at 5 x 10^5 cells/well of a 48-well plate overnight, then incubated for 45 minutes at 37°C with either 250µl DMEM, or 250µl MEEM containing 5.8mM Ca^{2+}/0.86mM Pi. Cells were washed twice with PBS and 500µl DMEM containing 0.2% FCS added. Cells were incubated at 37°C for 1hour or 23 hours, and media analysed by TNF-α ELISA. Values represent mean ± S.E. of n=3 experiments, each performed in triplicate. *** = significant difference, $P<0.001$, compared with cells incubated for 23 hours following treatment with DMEM.

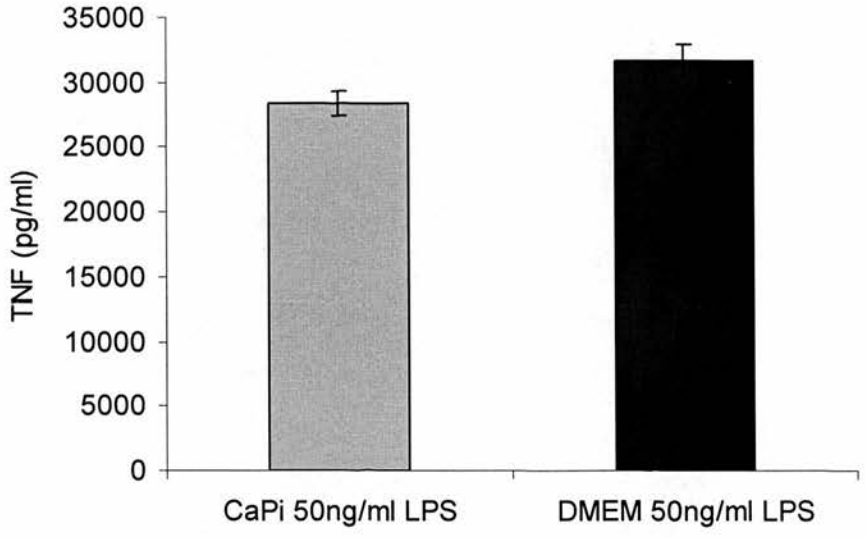


Figure 5. Incubation of RAW 264.7 macrophages with calcium phosphate (CaPi) has little effect on their inflammatory response to LPS

RAW 264.7 murine macrophages were seeded at 5 x 10^5 cells/well of a 48-well plate overnight, then incubated for 45 minutes at 37°C with either 250µl DMEM, or 250µl MEEM containing 5.8mM Ca^{2+}/0.86mM Pi. Cells were washed twice with PBS and 500µl DMEM containing 0.2% FCS added. Following 23 hours incubation, cells were stimulated with 50ng/ml *E. coli* O55:B5 LPS for 4 hours at 37°C; media were analysed by TNF-α ELISA. Values represent mean ± S.E. of n=3 experiments, each performed in triplicate.

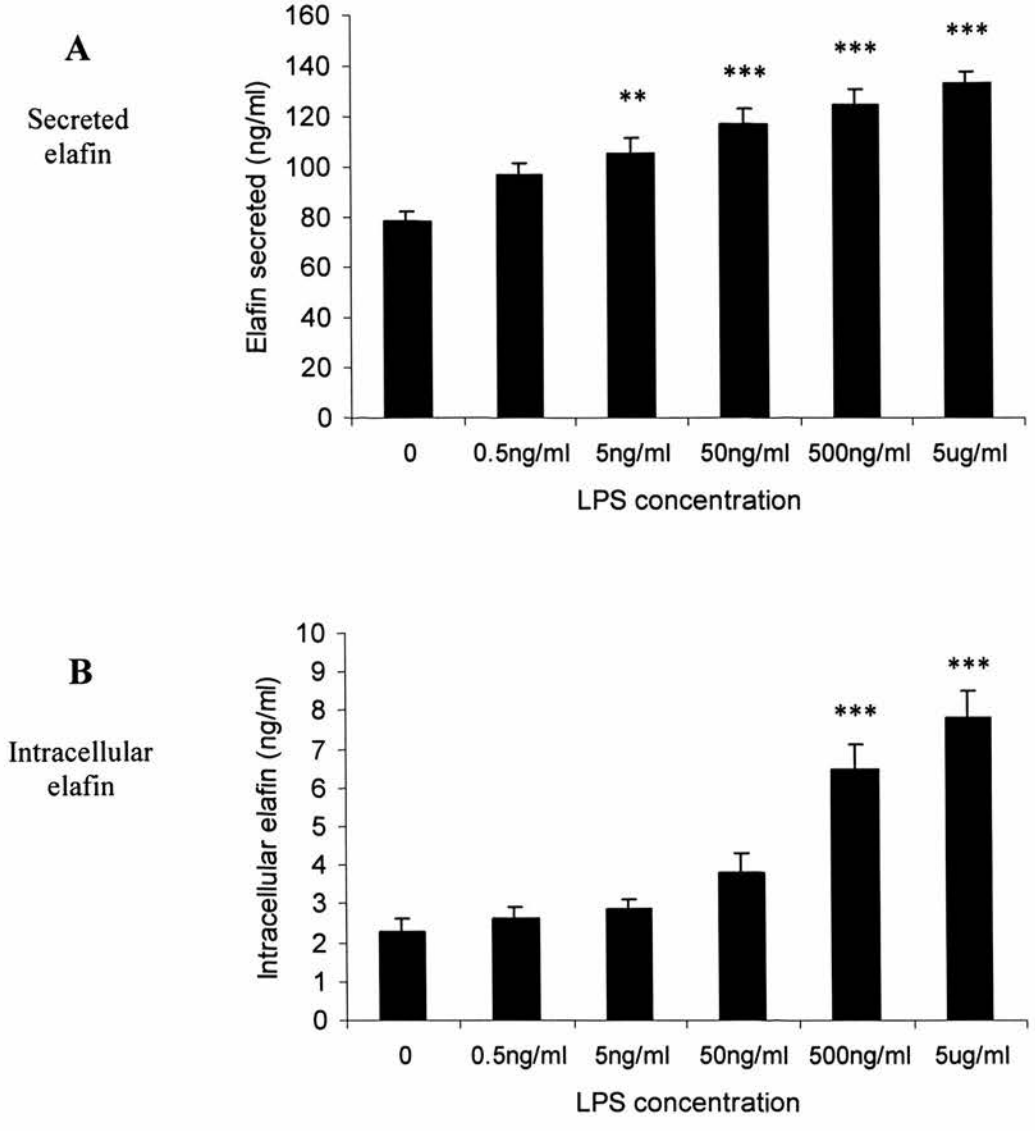


Figure 6. LPS upregulates elafin production by Ad-elafin-infected RAW 264.7 macrophages

RAW 264.7 murine macrophages were seeded at 5 x 10^5 cells/well of a 48-well plate overnight. Cells were then infected with Ad-elafin (moi 100) for 45 minutes at 37°C, in 250µl MEEM containing 5.8mM Ca^{2+}/0.86mM Pi. Cells were washed twice with PBS and 500µl DMEM containing 0.2% FCS added. Following 23 hours incubation, cells were stimulated with increasing concentrations of *E. coli* O55:B5 LPS for 4 hours at 37°C. Media were analysed by elafin ELISA to measure levels of secreted elafin (A). Alternatively, cells were lysed to obtain total protein and lysates analysed by ELISA for intracellular elafin (B). Values represent mean ± S.E. of n=3 experiments, each performed in triplicate. ** = significant difference, $P<0.01$, compared with baseline (0 LPS). *** = significant difference, $P<0.001$, compared with baseline (0 LPS).

Figure 6 demonstrates that 4-hour incubation of LPS with Ad-elafin-infected cells caused a dose-responsive increase in elafin production, both extracellularly and intracellularly. This effect was very significant at higher concentrations of LPS, although as low as 5ng/ml LPS induced a significant increase in the concentration of elafin secreted. While levels of secreted elafin could be significantly augmented by lower LPS concentrations than intracellular (Figure 6A), intracellular elafin was more sensitive to up-regulation in terms of 'fold-increase' (for example, an approximately four-fold increase at 5μg/ml LPS) (Figure 6B).

5.2.3. Inflammatory response of RAW 264.7 murine macrophages to infection with adenovirus constructs

Prior to investigating the effects of elafin overexpression on the TNF-α response of macrophages to LPS, it was necessary to consider the inflammatory response of cells to infection with adenovirus constructs in the absence of additional stimulation. For the purpose of the experiments described in the following section, several adenovirus vectors were used to provide informative controls. In addition to Ad-elafin, infections were carried out using the following vectors: Ad-IκB, encoding the gene for an NF-κB super-repressor mutant of the cytoplasmic protein IκBα, known to play an essential role in the regulation of NF-κB activation (which subsequently regulates TNF-α production (Shakhov *et al.*, 1990)) (Baldwin, 1996; Jobin *et al.*, 1998); Ad-dl70/3, an empty adenovirus vector which does not encode a transgene; Ad-m-eotaxin, encoding the cDNA for the murine CC chemokine eotaxin, an 8.4kDa secreted protein which acts as a chemoattractant for inflammatory leukocytes, particularly eosinophils (Rothenberg *et al.*, 1995; Corrigan, 1999); and finally Ad-mSLPI, which encodes the cDNA for murine SLPI, the other member of the antileukoprotease superfamily of proteinase inhibitors. The Ad-mSLPI construct was regarded as a potential control vector for the following LPS stimulation assays, since several previous investigations have suggested that SLPI can exert anti-inflammatory effects intracellularly (Zhu *et al.*, 1999; Mulligan *et al.*, 2000; Taggart *et al.*, 2002). These studies will be discussed in more depth in the 'Discussion'

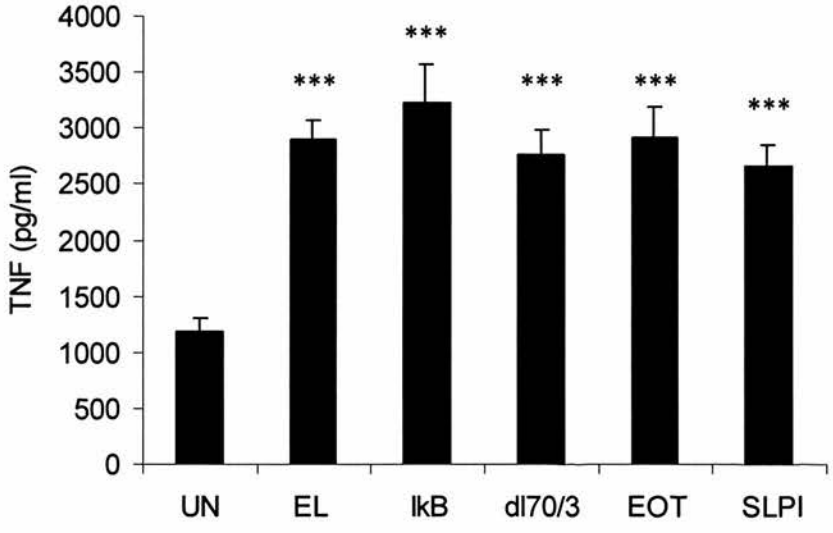


Figure 7. Infection of RAW 264.7 macrophages with adenoviral constructs induces a significant inflammatory response, compared with uninfected cells

RAW 264.7 murine macrophages were seeded at 5 x 10^5 cells/well of a 48-well plate overnight, then infected with Adenovirus constructs (moi 100) for 45 minutes at 37°C in 250µl MEEM containing 5.8mM Ca^{2+}/0.86mM Pi. Cells were washed twice with PBS, 500µl DMEM containing 0.2% FCS added and cells incubated for 23 hours at 37°C. Media were then analysed by TNF-α ELISA. Key to virus-infected cells: UN=uninfected, EL=Ad-elafin, IkB=Ad-IκB, dl70/3=Ad-dl70/3, EOT=Ad-m-eotaxin, SLPI=Ad-mSLPI. Values represent mean ± S.E. of n=3 experiments, each performed in triplicate. *** = significant difference, $P<0.001$, compared with uninfected cells.

section of this chapter. However, it should be noted that effects of SLPI using this system have not previously been documented.

Adenoviral infection of RAW 264.7 macrophages with all constructs induced a significant inflammatory response, in comparison to uninfected cells (Figure 7). TNF-α release was measured after incubation of macrophages with viral constructs at moi 100 or with vehicle alone, followed by incubation for 23 hours; this incubation represents the same period for which cells would be left to express transgene before being stimulated with LPS in the experiments to follow. However, while viral infection with Ad constructs elicited an inflammatory response *per se*, the levels of response induced by the different vectors were similar and, indeed, non-significant when compared together statistically. In general, viral infection induced around a 2.5-fold increase in TNF-α secretion (Figure 7).

5.2.4. The effect of elafin gene augmentation on the inflammatory response of RAW 264.7 murine macrophages to LPS

The effects of Ad-elafin infection on the LPS-mediated TNF-α response of RAW 264.7 cells were investigated in a series of linked experiments (Figures 8 and 9). Firstly, cells were virally infected, incubated for 23 hours to allow expression of transgene and then stimulated with 50ng/ml LPS in their conditioned media (Figure 8A). The TNF-α responses of Ad-dl70/3- (empty vector) and Ad-m-eotaxin-infected cells were similar to that observed with uninfected cells. Ad-m-eotaxin was chosen as a control since the product of this transgene is a secreted protein of similar molecular weight to elafin (8.4 kDa and 9.9 kDa respectively), and this chemokine was detectable by ELISA (data not shown); it was shown, however, to have no effect on LPS activation of these cells. In contrast, Ad-elafin-, Ad-IκB- and Ad-mSLPI-infected cells were significantly hyporesponsive to LPS stimulation in the presence of their conditioned media (Figure 8A). Interestingly, these cells exhibited an even more profound degree of LPS-hyporesponsiveness when conditioned media were replaced with fresh media just prior to addition of LPS (Figure 8B). This suggests that products secreted by cells during the 23-hour incubation, such as sCD14 or LBP

perhaps, may accelerate the response to LPS and mask to a certain extent the effects of the viral transgenes. The average percentage decrease in TNF-α secretion by Ad-elafin-, Ad-IκB- or Ad-mSLPI-infected cells, as compared with uninfected cells, is provided above each bar. As shown by both Figures 8A and 8B, the most potent inhibitor of TNF-α secretion was the NF-κB super-repressor Ad-IκB, while the anti-inflammatory effects of Ad-elafin and Ad-mSLPI were comparable and highly significant.

In order to investigate whether secreted (transgene-derived) products of adenovirally-infected cells could suppress the response of uninfected cells to LPS, conditioned media were removed from Ad-infected wells and added to washed 'naïve' cells prior to addition of LPS (Figure 9). For these assays, conditioned media were removed from Ad-treated cells either 4 hours or 23 hours post-infection; media derived from 4-hour incubations permitted us to investigate whether the effects observed in Figure 8B could be attributed to the effects of peptides secreted during LPS stimulation. Conditioned media from Ad-elafin-infected cells had no effect on the LPS-mediated TNF-α response of uninfected cells, whether taken from cells 23 hours (Figure 9A) or 4 hours (Figure 9B) post-infection. These results strongly suggest that secreted elafin did not contribute to the LPS-hyporesponsive phenotype of Ad-elafin-infected cells, observed in Figure 8, and that elafin retained intracellularly may play a role in this effect. Additionally, conditioned media from Ad-IκB- and Ad-mSLPI-infected cells failed to confer LPS-hyporesponsiveness to uninfected cells (Figure 9); while the product of the Ad-IκB transgene is retained intracellularly, Ad-mSLPI-infected cells produce a secreted protein, which was found to have no effect on LPS activation. Similarly, and as expected, conditioned media derived from the negative control vectors Ad-dl70/3 and Ad-m-eotaxin had no effect on TNF-α secretion.

Figure 10 demonstrates the elafin levels produced by Ad-elafin-infected cells during the course of these experiments, measured both extracellularly (Figure 10A) and intracellularly (Figure 10B). During 23-hour incubation post-infection and subsequent 4-hour incubation with LPS, Ad-elafin-infected cells secreted around

100ng/ml elafin (Figure 10A, bar 1); around 90% of this was removed by washing elafin-conditioned media from cells prior to LPS stimulation (Figure 10A, bar 2). Therefore, approximately 100ng/ml (10nM) elafin was present in the media bathing Ad-elafin-infected cells during LPS stimulation in the experiments described by Figure 8A, and this was reduced to around 10ng/ml (1nM) in the experiments described by Figure 8B. These findings taken alone could suggest that extracellular elafin was responsible for dampening macrophage responses to LPS in these assays, and that the effects of elafin are more pronounced at lower concentrations. However, as shown in Figure 9, analogous elafin-conditioned media from Ad-elafin-infected cells failed to confer LPS-hyporesponsiveness to uninfected cells. Bars 3 and 4 of Figure 10A indicate that elafin levels in media transferred to uninfected cells were either approximately 70ng/ml (7nM) or 6ng/ml (0.6nM). The discrepancies between bars 1 and 3, and bars 2 and 4 respectively, are likely due to LPS upregulation of the transgene in Ad-elafin cells during the 4-hour LPS incubation period; this upregulation cannot occur in the uninfected cells (bars 3 and 4).

In terms of intracellular elafin, the technique used to measure these levels may have taken into account elafin on the cell surface, or else some cells were removed by washing, since intracellular elafin was lower after washing the cells (Figure 10B, comparing bars 1 and 2). Moreover, elafin was detected in the lysates of uninfected cells to which had been added Ad-elafin-conditioned media (Figure 10B, bars 3 and 4). It is unclear if this represents elafin which was attached to the cell surface on lysis, or if the cells internalised elafin from the surrounding milieu. As mentioned previously, no murine homologue of elafin has been identified to date, and no murine protein has been observed to cross-react with the anti-elafin antibody used in these experiments.

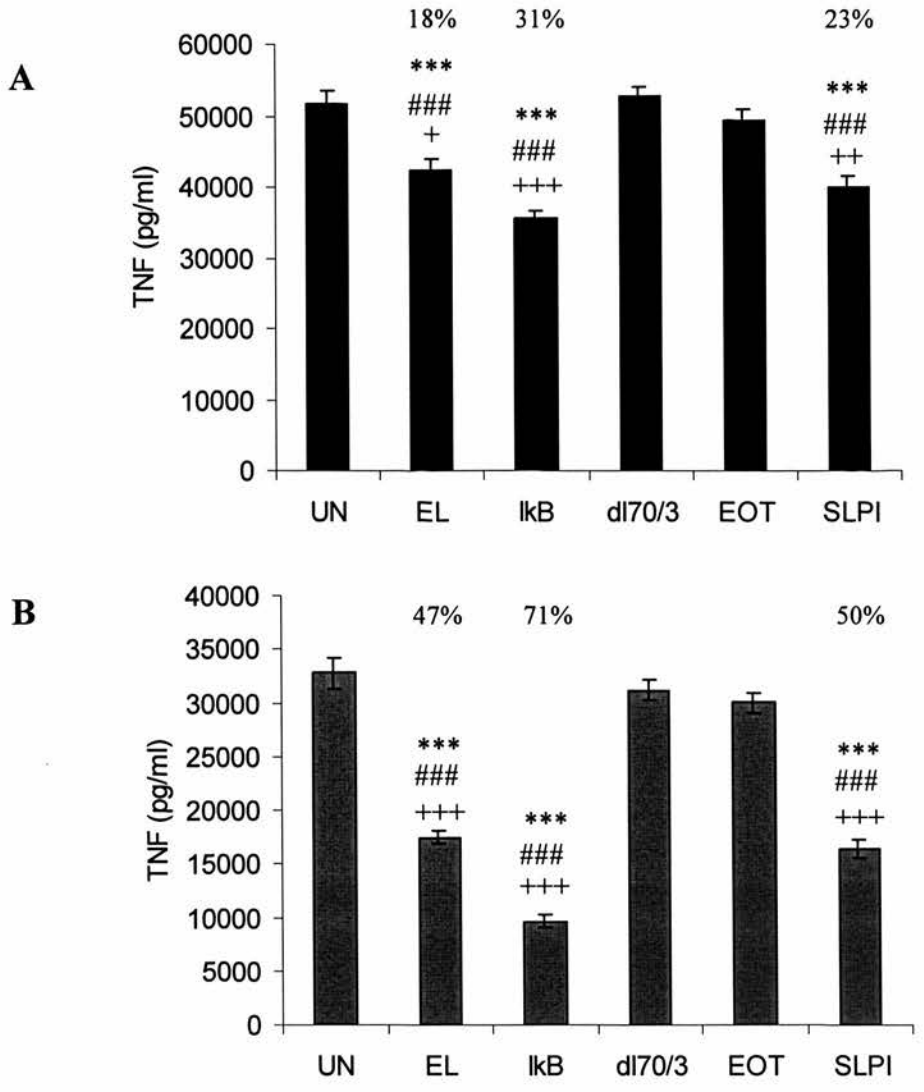


Figure 8. Ad-elafin-infected RAW 264.7 macrophages are hyporesponsive to LPS, and this effect is more pronounced when conditioned medium is replaced with fresh medium prior to stimulation

RAW 264.7 murine macrophages were seeded at 5 x 10^5 cells/well of a 48-well plate overnight, then infected with Ad constructs (moi 100) for 45 minutes at 37°C in 250µl MEEM containing 5.8mM Ca^{2+}/0.86mM Pi. Cells were washed twice with PBS, 500µl DMEM containing 0.2% FCS added and cells incubated for 23 hours at 37°C. 50ng/ml *E. coli* O55:B5 LPS was then incubated with cells for 4 hours in their conditioned media (A), or alternatively conditioned media were replaced with fresh DMEM/0.2% FCS prior to addition of LPS (B). Media were analysed by TNF-α ELISA. Key to virus-infected cells: UN=uninfected, EL=Ad-elafin, IkB=Ad-IκB, dl70/3=Ad-dl70/3, EOT=Ad-m-eotaxin, SLPI=Ad-mSLPI. Values represent mean ± S.E. of n=3 experiments, each performed in triplicate. *** = significant difference, P<0.001, compared with uninfected cells. ### = significant difference, P<0.001, compared with Ad-dl70/3-infected cells. + = significant difference, P<0.05, compared with Ad-EOT-infected cells. ++ = significant difference, P<0.01, compared with Ad-EOT-infected cells. +++ = significant difference, P<0.001, compared with Ad-EOT-infected cells. Figures above bars represent average % decrease in TNF-α secretion, as compared with uninfected cells.

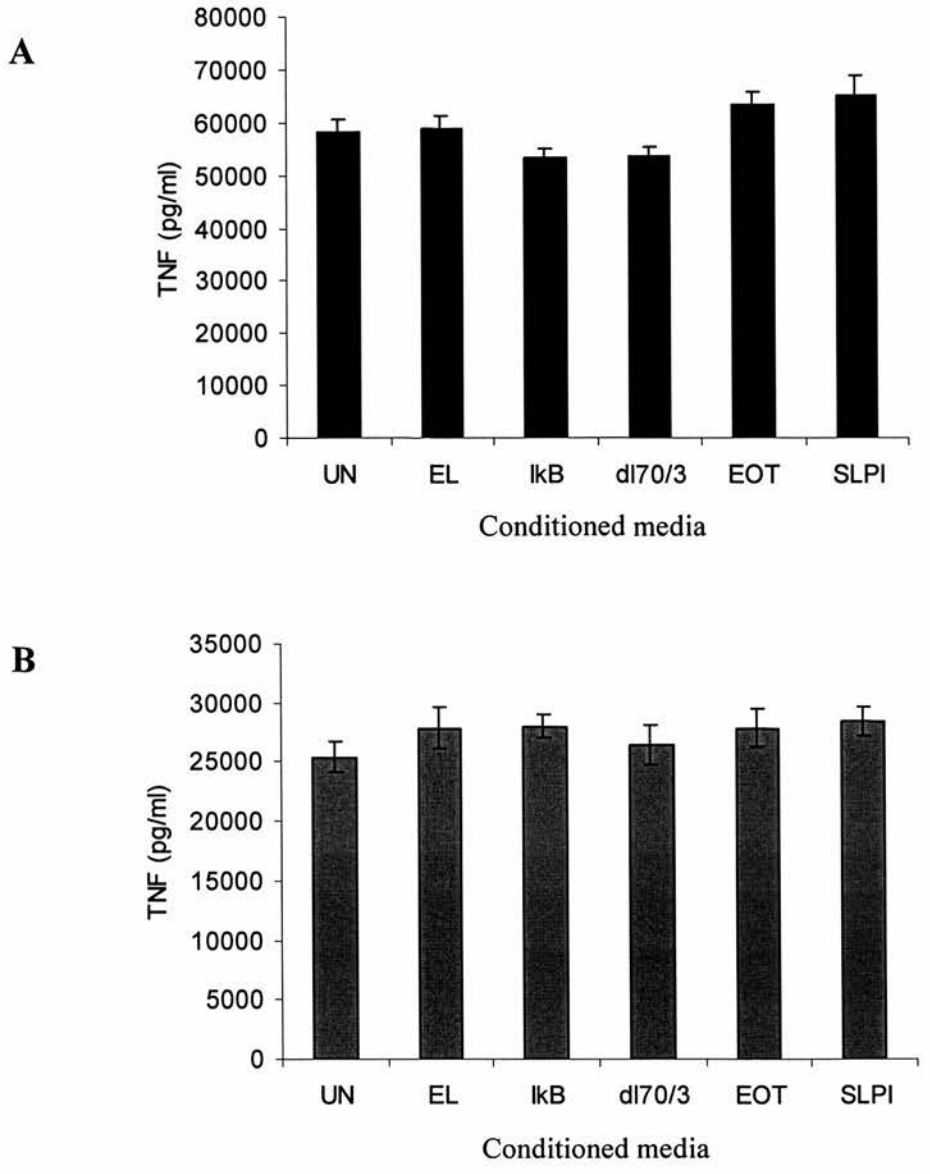


Figure 9. Conditioned medium from Ad-elafin-infected RAW 264.7 macrophages does not confer LPS-hyporesponsiveness to uninfected cells

RAW 264.7 macrophages were plated out and infected with Adenovirus constructs (moi 100) as per Figure 8. Conditioned media were removed from cells 23 hours and 4 hours post-infection, and these media were added to 5 x 10^5 washed, uninfected cells/well of a 48-well plate. 50ng/ml *E. coli* O55:B5 LPS was then incubated with cells for 4 hours in these conditioned media, and media were analysed by TNF-α ELISA. (A) 23-hour conditioned media added to uninfected cells prior to LPS stimulation. (B) 4-hour conditioned media added to uninfected cells prior to LPS stimulation. Key to conditioned media added to uninfected cells: UN=uninfected, EL=Ad-elafin, IkB=Ad-IκB, dl70/3=Ad-dl70/3, EOT=Ad-m-eotaxin, SLPI=Ad-mSLPI. Values represent mean ± S.E. of n=3 experiments, each performed in triplicate.

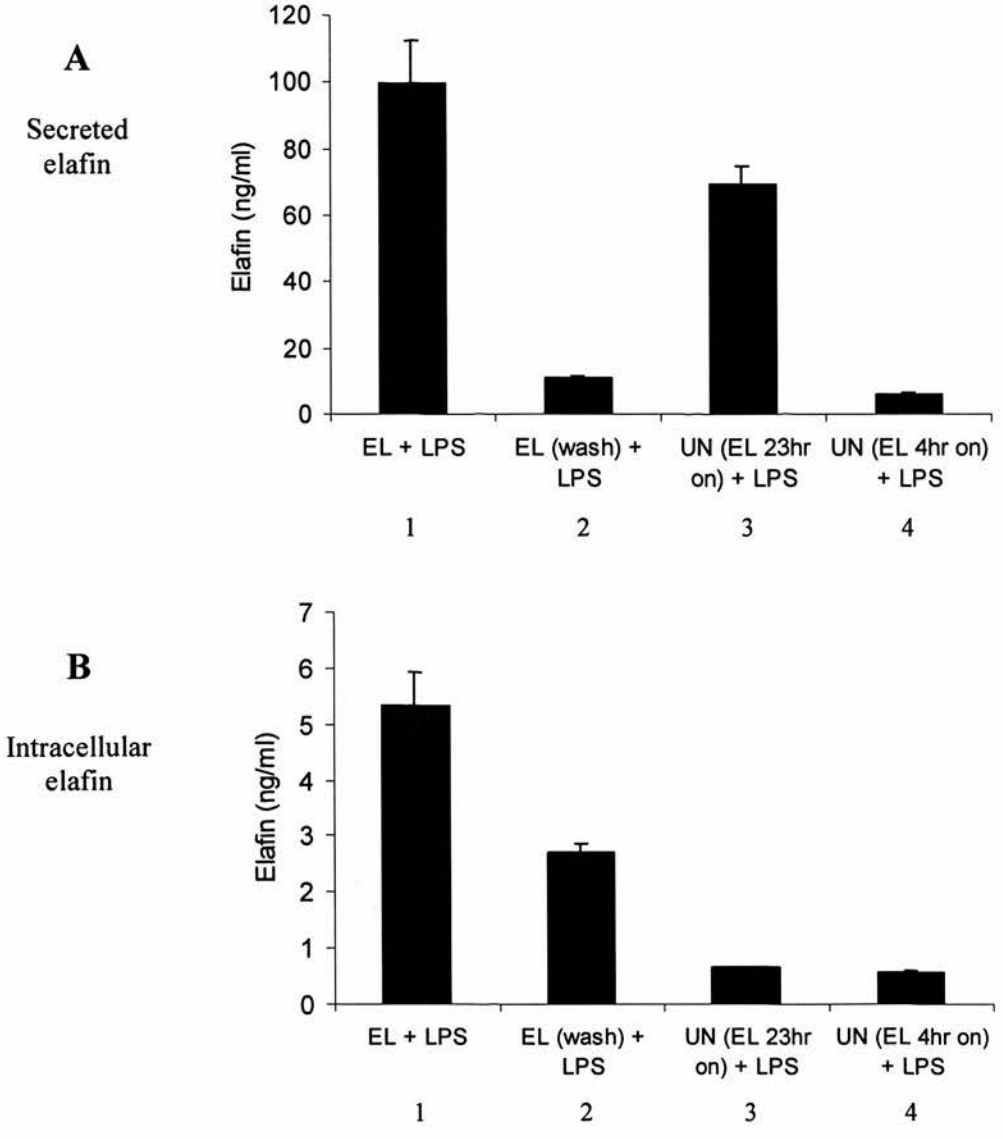


Figure 10. Elafin levels produced by RAW 264.7 macrophages during LPS stimulation assays

RAW 264.7 murine macrophages were seeded at 5 x 10^5 cells/well of a 48-well plate overnight. Cells were then infected with Ad-elafin (moi 100) for 45 minutes at 37°C, in 250µl MEEM containing 5.8mM Ca^{2+}/0.86mM Pi. Cells were washed twice with PBS, 500µl DMEM containing 0.2% FCS added and cells incubated at 37°C for 23 hours or 4 hours (producing 'elafin-conditioned media'). Cells were then incubated for 4 hours with 50ng/ml *E. coli* O55:B5 LPS, either in their 23-hour elafin-conditioned media (bar 1) or following washing with PBS and addition of fresh medium (bar 2). Alternatively, elafin-conditioned media were removed from Ad-infected wells and added to uninfected, washed cells prior to LPS stimulation (bars 3 and 4).
Media were analysed by elafin ELISA to measure levels of secreted elafin (A). Alternatively, cells were lysed to obtain total protein and lysates analysed by ELISA for intracellular elafin (B). UN=uninfected cells, EL=Ad-elafin-infected cells. Values represent mean ± S.E. of n=3 experiments, each performed in triplicate.

5.3. DISCUSSION

The principal novel observation arising from this work is that infection of murine macrophages with an adenovirus vector encoding elafin suppresses their vigorous pro-inflammatory response to bacterial LPS (Figure 8). Moreover, two prominent findings suggest that this anti-inflammatory activity is independent of the effects of extracellular elafin. Firstly, Ad-elafin-infected cells retained their LPS-hyporesponsive phenotype even after elafin-conditioned media were removed prior to stimulation, and indeed suppression of responses was more pronounced under these conditions (Figure 8B). Secondly, conditioned media from Ad-elafin-infected cells failed to dampen the inflammatory response of uninfected macrophages to LPS (Figure 9).

Discussion of these data will focus on the efficiency of adenovirus-mediated gene transfer to murine macrophages, the effects of LPS on elafin production by these cells, and on the effects of elafin gene augmentation on their inflammatory response to LPS.

5.3.1. Adenoviral infection of the murine macrophage cell line RAW 264.7

Much of the data described in the first part of this chapter concerns the establishment of a set of optimal conditions for adenoviral infection of the RAW 264.7 macrophage cell line, and consequently expression of the elafin transgene. Murine cells were studied in view of the facts that no murine homologue of elafin has been identified to date, and that our laboratory has observed no cross-reaction of the anti-human elafin antibody with any mouse product; thus we could study the effects of introducing an entirely foreign gene (human elafin) into murine cells. We chose to use macrophages as a target for adenoviral gene transfer in the context of an *in vitro* model of LPS-induced inflammation, since this cell type is pivotal as the major producer of the prototypic inflammatory cytokine TNF-α, which mediates many of the immunopathological features of septic shock (Beutler *et al.*, 1985; Dinarello, 1997; Cohen, 2002). Macrophages are known to be one of a number of cell types, including B and T lymphocytes, dendritic cells and fibroblasts, that are not

easily infected by adenovirus vectors due to a low level of expression of the adenovirus receptor CAR (Hidaka *et al.*, 1999; Kaner *et al.*, 1999; Schmidt *et al.*, 2000; Stockwin *et al.*, 2002). Several studies have demonstrated that such low levels of adenoviral infection efficiency can be surmounted by overexpression of CAR in the target cell (Hidaka *et al.*, 1999; Schmidt *et al.*, 2000; Nalbantoglu *et al.*, 2001; Walters *et al.*, 2001; Stockwin *et al.*, 2002).

For the purpose of our studies, we chose to investigate the value of using CaPi precipitates to enhance non-specific binding of adenovirus to macrophages, and consequently to improve gene transfer of elafin to these cells. This technique has previously been used to augment adenoviral gene transfer to airway epithelia both *in vitro* and *in vivo* (Fasbender *et al.*, 1998; Walters *et al.*, 2001).

In this study, infection of macrophages with adenovirus was also shown to be markedly enhanced by introducing virus to cells in the presence of CaPi co-precipitates. Indeed, this approach facilitated an infection efficiency around 100% at a viral moi of 100, as visualised using the Ad-lacZ reporter construct (Figure 1). Moreover, increasing the efficiency of viral uptake also meant that a considerable level of elafin overexpression could be achieved in these macrophages (Figures 2 and 3). The findings of this investigation are very similar to those of Fasbender *et al.* (1998) with regards to the observation that optimal transgene expression was obtained on addition of 4mM Ca^{2+} to form CaPi precipitates (Figure 2). These findings suggest that perhaps the stoichiometry of Ca^{2+} to Pi may be optimal for precipitate formation at this concentration. While the macrophages appeared to be directly activated by CaPi precipitates, likely due to phagocytosis of the particles thus inducing release of TNF-α (Figure 4), this inflammatory response did not lead to any dampening of the cells' subsequent pro-inflammatory response to LPS (Figure 5).

An important point to note is that the levels of elafin measured from Ad-elafin-infected cells could be extremely variable from assay to assay. In general, however, macrophages infected with Ad-elafin at moi 100 secreted between 100-

300ng/ml elafin (around 10nM-30nM) and retained around 2-40ng/ml (0.2nM-4nM) intracellularly (Figures 2, 3, 6 and 10). The levels of elafin secreted by Ad-elafin-infected macrophages were therefore within a similar range to those levels of synthetic elafin peptide (10nM-100nM) found to be effective in down-regulating the effects of LPS in serum-containing medium in Chapter 4. The lack of an observed effect of secreted elafin in this chapter, and the potential importance of measurable levels of elafin retained intracellularly by Ad-elafin-infected cells, will be discussed in section 5.3.3.

To date, two other studies have measured elafin levels produced by Ad-elafin-infected macrophages. Simpson *et al.* (2001a) infected human monocyte-derived macrophages with Ad-elafin at moi 50 without CaPi precipitates, and reported maximum secreted elafin levels of 11.6ng/ml (of which up to around 1.5ng/ml may be attributable to endogenous human elafin). The same study also reported adenoviral infection efficiency of macrophages of approximately 16%, in agreement with similarly low rates of infection reported by others (Haddada *et al.*, 1993; Schneider *et al.*, 1997; Worgall *et al.*, 1999). Recent work carried out in our laboratory, concurrently with studies described here, demonstrated that pre-complexing Ad-elafin with the cationic liposome lipofectamine could also enhance adenoviral gene delivery to human (monocyte-derived) macrophages (Henriksen *et al.*, 2004); the study by Henriksen *et al.* (2004) reported levels of elafin secretion similar to those described here, using Ad-elafin at moi 100. These techniques thus represent a vast improvement on previous protocols for adenovirus-mediated gene transfer to macrophages.

5.3.2. The effect of LPS on elafin production by Ad-elafin-infected murine macrophages

Prior to investigating the effects of elafin overexpression on the response of macrophages to LPS, it seemed important firstly to establish how LPS stimulation would affect the elafin transgene in adenovirally-infected cells.

On consideration of the work of Simpson *et al.* (2001a), which reported that LPS can upregulate the elafin transgene *in vitro* (in A549 cells and human macrophages) and *in vivo* (murine lungs), it also seemed valuable to examine the effects of LPS on elafin production by Ad-elafin-infected murine macrophages in this system. This earlier work had been prompted by the knowledge that LPS could stimulate the MCMV promoter, due to the presence of LPS-responsive elements (Loser *et al.*, 1998); the MCMV promoter is incorporated upstream of the elafin transgene in the Ad-elafin construct (Sallenave *et al.*, 1998). Simpson *et al.* (2001a) demonstrated that 24-hour incubation of A549 cells with supraphysiological concentrations of LPS from 1-100μg/ml significantly augmented elafin secretion, most notably by 2.5-fold at 100μg/ml; although elafin secretion by macrophages was also upregulated by LPS in their study, values did not reach significance.

In this investigation, upregulation of the elafin transgene was demonstrated over a 4-hour incubation period, at much lower concentrations of LPS (Figure 6). Secretion of elafin was significantly augmented by as little as 5ng/ml LPS, while significant increases in intracellular elafin were observed on addition of 500ng/ml LPS or more (Figure 6); these findings suggest that elafin may be produced by cells predominantly as a secretory protein, and that elafin secretion following promoter activation is sensitive to low levels of LPS. In the peripheral blood of healthy humans, only small serum concentrations of around 3-10 pg/ml LPS are detectable (Casey *et al.*, 1993; Opal *et al.*, 1999). However, elevated systemic LPS levels with concentrations exceeding 500pg/ml are characteristic in patients with severe sepsis (Opal *et al.*, 1999). As shown in Figure 6, 0.5ng/ml (500pg/ml) LPS also elicited an increase in elafin secretion, although this was not statistically significant. It is therefore feasible that elafin production by Ad-elafin-infected macrophages may be upregulatable by patho-physiologically relevant concentrations of LPS, and in this regard could provide a negative feedback mechanism in inflammatory conditions such as sepsis.

5.3.3. The effect of elafin gene augmentation on the inflammatory response of RAW 264.7 murine macrophages to LPS

Before studying the effects of elafin overexpression on the LPS-mediated activation of macrophages, the response of these cells to adenoviral infection in the absence of further stimulation was investigated. Adenoviral infection of macrophages with all constructs induced a highly significant release of TNF-α (Figure 7). However, an important finding for our studies was that the differences between the response to each construct were non-significant, and so subsequent findings could be analysed in the absence of concerns surrounding baseline responses to adenoviral infection. The observation that adenoviral infection of macrophages was associated with significant TNF-α secretion came as little surprise, since macrophages are known to internalise adenovirus and initiate an inflammatory immune response (Kuzmin *et al.*, 1997; Worgall *et al.*, 1997). In spite of internalisation and clearance of adenovirus by macrophages *in vivo*, it is important to note that macrophage expression of adenoviral transgene is in fact feasible *in vivo* over a period of several days (Danel *et al.*, 1998; Worgall *et al.*, 1999).

The most edifying findings of this chapter were obtained from data shown in Figures 8 and 9. Ad-elafin-infected macrophages were significantly hypo-responsive to LPS, as compared with uninfected cells or with cells that had been infected with the control constructs Ad-dl70/3 (empty vector) or Ad-m-eotaxin.

As mentioned in the 'Results' section, the eotaxin construct was chosen for these studies as it encodes a secreted protein of very similar molecular weight to elafin, but which would perhaps not be expected to play a modulatory role in the LPS-induced activation of macrophages. The findings of this study indeed suggest that it does not interfere with the TNF-α response of macrophages to LPS (Figures 8 and 9). The same observation was made with cells infected with Ad-dl70/3, therefore it is apparent that adenoviral infection of macrophages *per se* does not provoke a general metabolic inhibition leading to suppression of TNF-α secretion.

Two further adenoviral constructs were employed as potential controls, and these were selected since it was anticipated that they would act as positive controls for inhibition of an inflammatory response. The Ad-IκB construct encodes a super-repressor of transcription factor NF-κB activity, a mutated IκBα resistant to phosphorylation and degradation; this vector has previously been shown to strongly inhibit NF-κB activity in human intestinal epithelial cells, resulting in down-regulation of multiple proinflammatory molecules (Jobin *et al.*, 1999). With particular relevance to the work described here, it is known that LPS-induced TNF-α production by macrophages is regulated by NF-κB (Shakhov *et al.*, 1990). In accordance, infection of the RAW 264.7 macrophage cell line with Ad-IκB significantly suppressed the TNF-α response of these cells to LPS stimulation (Figure 8). As expected, no similar effects were observed on transfer of Ad-IκB-conditioned media to uninfected cells since the anti-inflammatory activity of IκB results exclusively from its inhibition of NF-κB in the cytoplasm (Baeuerle and Baltimore, 1988) (Figure 9).

The second 'positive' control construct used in these assays was an adenovirus vector encoding murine SLPI (Ad-mSLPI). While SLPI overexpression has previously been shown to inhibit the TNF-α response of macrophages to LPS (Jin *et al.*, 1997; Zhu *et al.*, 1999), the system used to augment SLPI here is novel, and therefore the findings of this study also add to the knowledge surrounding functions of this molecule. The secretory nature of SLPI would suggest that it may act as an extracellular mediator to modulate macrophage activation by LPS. However, as discussed in Chapter 4, separate studies have failed to demonstrate any role for extracellular SLPI in down-regulating LPS-mediated TNF-α secretion by macrophages (Zhang *et al.*, 1997; Sano *et al.*, 2003); another group demonstrated that recombinant SLPI also failed to attenuate macrophage nitrite production in response to LPS (Zhu *et al.*, 1999). One possible explanation for these findings could relate to the importance of pre-incubating peptide with LPS prior to cellular stimulation (suggesting unfavourable LPS-binding kinetics), since this step was found to be critical in the modulation of LPS activity by elafin in Chapter 4. In this regard, it is interesting to note that conditioned media from neither Ad-elafin- nor

Ad-mSLPI-infected cells could dampen the TNF-α response of uninfected macrophages to LPS (Figure 9). Figure 10 provides data pertaining to measured elafin levels produced by Ad-elafin-infected cells, and it is notable that secreted elafin concentrations were around 100ng/ml (approximately 10nM). Pre-incubation of 10nM elafin peptide with LPS prior to macrophage stimulation was shown to significantly down-regulate TNF-α release in analogous culture conditions in Chapter 4 (Figure 5B). Given that incubation of peptide inhibitor with LPS prior to stimulation appears to be a key determinant of this effect, it may have also been informative to pre-incubate conditioned media with LPS before addition to cells; the execution of such experiments was unfortunately prohibited by time constraints.

Nonetheless, as alluded to in the 'Results' section, a number of previous studies have shown that SLPI is capable of exerting anti-inflammatory effects intracellularly (Zhu *et al.*, 1999; Mulligan *et al.*, 2000; Taggart *et al.*, 2002). Accordingly, Ad-mSLPI-infected macrophages were found to be significantly hypo-responsive to LPS in this study, and the magnitude of inhibition of TNF-α release closely paralleled that induced by Ad-elafin infection (Figure 8). In the case of cells overexpressing elafin or SLPI (or indeed IκB), the inhibition of TNF-α release was more profound when conditioned media were removed and replaced with fresh media prior to LPS stimulation; this could be due to the removal from the milieu of cell-derived LPS-catalysing molecules such as LBP, and provides further evidence to suggest that extracellular elafin and SLPI play no part in suppression of the TNF-α response in these assays.

Appendix V at the end of this chapter provides a diagrammatic representation of the proposed LPS signalling pathway, as previously discussed in Chapter 1. The pathway leading to activation of transcription factor NF-κB may represent the most likely candidate for inhibition by elafin (particularly on consideration of studies performed using SLPI, discussed below), and is a critical pathway for LPS-mediated TNF-α production (Shakhov *et al.*, 1990; Baeuerle and Henkel, 1994; Eigler *et al.*, 1997; Mannel and Echtenacher, 2000). However, other intracellular pathways downstream of TRAF6, involving mitogen-activated protein kinases (MAPKs) such

as Jun N-terminal kinase (JNK), p38 and extracellular signal-related kinase (ERK), and the transcription factor activated protein 1 (AP-1), may also be involved in the TNF-α response to LPS (Cao *et al.*, 2002; Kiemer *et al.*, 2002; Comalada *et al.*, 2003; Marshall, 2003). Potentially, elafin could also interfere with phosphorylation of any of these MAPKs, causing inhibition of downstream signalling and thus inhibition of AP-1 activation. The diagram in Appendix V also includes potential points of elafin interaction with the pathway (including those proposed in Chapter 4), some of which are still to be discussed here.

In evaluating the anti-inflammatory effects of Ad-elafin demonstrated in Figure 8 of this chapter, it is perhaps most instructive, given the similarity between these two molecules, to firstly consider the findings obtained by others with SLPI.

Earlier work carried out by Jin *et al.* (1997) demonstrated that macrophages derived from a mouse line naturally resistant to LPS (C3H/HeJ) constitutively overexpress SLPI, in contrast with macrophages that display sensitivity to LPS, such as those derived from the C3H/HeN mouse line. This evidence for an important role for SLPI in conferring LPS-unresponsiveness in C3H/HeJ mice has been eclipsed by the discovery in this strain of a point mutation in the intracellular region of the *Tlr4* gene, causing abrogation of downstream signalling and therefore LPS-hyporesponsiveness (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999); this discovery was confirmed in *Tlr4* knockout mice (Hoshino *et al.*, 1999). TLR4, the product of this gene, is now recognised as the signal-transducing receptor for LPS in mammalian species (reviewed by Kopp and Medzhitov, 2003; Takeda *et al.*, 2003). Interestingly, in the Jin *et al.* (1997) study, LPS hyporesponsiveness could be restored by interferon-γ (IFN-γ), and a recent study has demonstrated that IFN-γ enhances surface expression of TLR4 in human monocytes and macrophages (Bosisio *et al.*, 2002).

A recent study by Sano *et al.* (2003) sought to further investigate the hypothesis of Jin *et al.* (1997) concerning a role for SLPI in the LPS-hyporesponsiveness of macrophages from C3H/HeJ mice, and concluded that SLPI

overexpression was not a factor. However, this study only investigated the effects of extracellular SLPI on LPS-induced TNF-α secretion, and therefore this conclusion cannot be drawn from their findings. Indeed, these observations (along with those made here) serve to strengthen the evidence for an intracellular role for SLPI, since the experiments carried out by Jin *et al.* (1997) also involved transfer of the SLPI gene to macrophages; transfection of macrophages with SLPI suppressed the LPS-induced activation of NF-κB and production of nitric oxide and TNF-α (Jin *et al.*, 1997). Interestingly, Jin *et al.* (1998) have also shown that transfection of macrophages with SLPI can inhibit nitric oxide production in response to gram-positive lipoteichoic acid.

Furthermore, an investigation carried out by Lentsch *et al.* (1999a) demonstrated, in an IgG immune complex rat model of lung injury (which induces a pathophysiology similar to sepsis), that exogenous SLPI reduced lung inflammation by reducing activation of NF-κB; inhibition of NF-κB activity by SLPI was associated with elevated levels of lung IκBβ (Lentsch *et al.*, 1999a). The anti-inflammatory effect of SLPI in this case was shown to be targeted at cell types other than macrophages, for example by decreasing neutrophil influx through blockade of expression of the adhesion molecule ICAM-1 by endothelial cells (Lentsch *et al.*, 1999a). More recently, work carried out using SLPI-deficient ($SLPI^{-/-}$) mice, which show a higher mortality rate from endotoxin shock than wild-type mice, has demonstrated that macrophages derived from $SLPI^{-/-}$ strains have increased NF-κB DNA binding activity and suppressed IκBβ expression during LPS stimulation (Nakamura *et al.*, 2003).

Several investigators have specifically suggested that SLPI may play a prominent intracellular role in dampening macrophage responsiveness to LPS, and that this may involve inhibition of the signal transduction pathway leading to NF-κB activation (see Appendix V). An important finding was that transfection of macrophages with a non-secretory (cytosolic) form of SLPI blocked their LPS-mediated release of TNF and nitrite (Zhu *et al.*, 1999). Moreover, a more recent study has demonstrated that SLPI can prevent LPS-induced NF-κB activation by

preventing degradation of the regulatory proteins interleukin-1 receptor-associated kinase (IRAK), IκBα (without affecting phosphorylation or ubiquitination) and IκBβ (Taggart *et al.*, 2002). This inhibition was shown to be dependent upon the antiproteinase activity of SLPI since an oxidised form of the molecule could not elicit these effects. The importance of antiproteinase activity is in agreement with the work of Mulligan *et al.* (2000), who have shown that SLPI mutants with reduced protease-inhibitory capacity (due to substitutions at Leu^{72}, known to be critical for binding of SLPI to serine proteases (Eisenberg *et al.*, 1990)) are less able to inhibit NF-κB activation than wild-type SLPI, in IgG immune complex-induced alveolitis in rats. This study found that the suppressive effects of SLPI were most closely linked to its trypsin-inhibitory activity (Mulligan *et al.*, 2000). The two latter groups have both hypothesised that SLPI's anti-inflammatory effects may thus be mediated by inhibition of intracellular proteases of the proteasome pathway, and subsequent inhibition of release and nuclear translocation of NF-κB.

The studies of Taggart *et al.* (2002) and Mulligan *et al.* (2000) both involved use of extracellular SLPI peptides, and these groups suggested that cellular internalisation of SLPI was a critical pre-requisite for its anti-inflammatory activity. Previous work has demonstrated that SLPI binds with high affinity to a receptor on the surface of monocytes and is internalised by these cells (McNeely *et al.*, 1997). The small size of molecules such as elafin and SLPI (9.9 kDa and 11.7 kDa respectively) may aid their access to the intracellular environment, since no evidence has been reported of internalisation of the larger (52 kDa) serine protease inhibitor α_1-PI. It would be interesting to perform experiments involving pre-incubation of elafin peptides with macrophages prior to LPS stimulation, since this may provide a more informative answer to the questions of whether elafin uptake takes place and to what degree, and whether this uptake has an effect on LPS signalling and macrophage activation. Although a low level of elafin was detected in the lysates of non-virally-infected cells that had been incubated with elafin-conditioned media (Figure 10B, bars 3 and 4), these levels did not correlate with the differing concentrations of elafin measured in media 23 hours or 4 hours post-infection (Figure 10A, bars 3 and 4); moreover, it is unclear whether elafin measured in lysates

had been taken up by cells, or was representative of elafin bound to the cell surface on lysis.

Chapter 4 demonstrated that exogenous elafin peptides could act to either up-regulate or down-regulate LPS-mediated TNF-α secretion, depending on the serum content of the surrounding milieu. In serum-containing conditions parallel with those investigated in this chapter, elafin exerted anti-inflammatory effects that required complexing of elafin with LPS prior to stimulation. While it is possible that these effects could, at least partly, be attributed to internalisation of elafin from medium and subsequent elafin activity intracellularly, the observation that extracellular elafin-LPS binding was critical suggests that this may not be the case. On the other hand, if a hitherto unidentified elafin receptor exists on the surface of myeloid cells, then it could be speculatively suggested that elafin-LPS complexes may be targeted to a pathway leading to dampening of inflammatory responses. It seems more likely that the anti-inflammatory effects of extracellular elafin observed in Chapter 4 result from direct binding of the peptide to LPS, and interference of the subsequent LPS interaction with molecules of the receptor complex, such as LBP, CD14 and the TLR4-MD-2 complex.

In any case, our study demonstrates that Ad-elafin infection of macrophages renders them hypo-responsive to LPS, and that this activity does not relate directly to elafin secreted by these cells. Thus, it is suggested that elafin overexpression may also modulate macrophages responses to LPS from within the cell. Similar to the findings obtained by others with SLPI (and corroborated here), elafin gene transfer to macrophages appears to interfere with the LPS signalling pathway regulating TNF-α release, and this inhibition is most likely to occur ultimately through inhibition of NF-κB activation (Shakhov *et al.*, 1990).

Indeed, a study by Henriksen *et al.* (2004) from our group, published during the writing of this chapter, has shown that adenovirus-mediated overexpression of elafin (and SLPI) can reduce TNF-α secretion by monocyte-derived macrophages in

response to LPS; moreover, these effects were associated with decreased activation of NF-κB, and concomitant inhibition of IκBα degradation (Henriksen *et al.*, 2004).

It is therefore eminently possible that elafin elicits anti-inflammatory effects in macrophages intracellularly by inhibiting degradation of IκB via the proteasome pathway. The aforementioned studies with SLPI have suggested the importance of its antiproteinase activity for exertion of anti-inflammatory activity, and the same may hold true for elafin. Inhibitors of the proteasome pathway have previously been shown to attenuate activation of NF-κB (Griscavage *et al.*, 1996; Haas *et al.*, 1998). With regards to the effects of proteinase inhibitors, the proteasome pathway is certainly known to possess trypsin and chymotrypsin-like activities, although the precise nature of the proteases involved in this process are not well characterised (Rivett, 1989; Hilt and Wolf, 1996). Although trypsin and chymotrypsin are not among the serine proteases which elafin specifically targets, it is possible that the action of other serine proteases, for example those with elastase-like activity, may be inhibited by elafin. In this way, elafin could potentially prevent proteasome-mediated degradation of IκB and thus inhibit the activation of NF-κB; proteolysis of IκB is known to be essential for induction of NF-κB, since phosphorylation and ubiquitination are insufficient to inactivate IκB, but are a necessary pre-requisite for targeting by the proteasome (Henkel *et al.*, 1993; Alkalay *et al.*, 1995; Lin *et al.*, 1995). It would thus be interesting to design similar experiments to those carried out using SLPI in the aforementioned studies, whereby elafin's antiproteinase activity could be obliterated or compromised.

Although the proteasome may be regarded as a major potential target of elafin in dampening macrophage responsiveness to LPS, it is also possible that these effects are mediated at another point in the intracellular pathway. Elafin may potentially bind and inactivate a component of the pathway upstream of the IκB/NF-κB complex, and this activity may not necessarily be dependent on protease inhibition. For example, the food derivative curcumin has been shown to block NF-κB activation by interfering with events upstream of IKK (Jobin *et al.*, 1999). It is

feasible that elafin could interfere with signal-transmitting adapter proteins such as MyD88, TIRAP or TRAF6, perhaps even by binding to them directly.

Furthermore, intracellular LPS receptors of the NOD protein family (described in depth in Chapter 1) may potentially be a target for elafin, assuming that internalised LPS may also be recognised by these proteins independently of TLR4. NOD2, for example, has been specifically demonstrated in macrophages (Pauleau and Murray, 2003). However, the role played by NOD receptors in recognition of LPS is controversial; for instance, macrophages with a complete disruption of TLR4 are non-responsive to LPS (Hoshino *et al.*, 1999), suggesting that if NOD proteins are indeed unique receptors for LPS, their activity must be downstream of TLR4.

Potentially, macrophages may also recognise internalised LPS via TLR4 in the cytosol, as demonstrated recently in intestinal and pulmonary epithelial cells (Hornef *et al.*, 2002; Hornef *et al.*, 2003; Guillot *et al.*, 2004). Intracellular TLR4-mediated recognition of LPS has not yet been demonstrated in macrophages, but could represent an additional target for inhibition by elafin.

The data in this chapter may also provide a further rationale to explain the findings of the recent study using transgenic mice expressing human elafin (Sallenave *et al.*, 2003). These mice express elafin cDNA under the control of the same MCMV promoter incorporated into the Ad-elafin vector used here. In that study, elafin transgenic mice produced lower levels of serum TNF-α than wild-type mice, in response to systemic or intratracheal LPS administration. Moreover, peritoneal macrophages from elafin transgenic mice were hyporesponsive to LPS *ex vivo*, in terms of TNF-α, MCP-1 and MIP-2 secretion (Sallenave *et al.*, 2003). The studies performed here aimed to use serum-containing conditions in order to model the effects of Ad-elafin on the inflammatory response of macrophages to LPS in the circulation. The findings described in this chapter appear to corroborate those of Sallenave *et al.* (2003), and further suggest that the dampening of systemic inflammatory responses in elafin transgenic mice may be attributable, at least in part, to intracellular effects of elafin on the LPS signalling pathway.

5.4. SUMMARY

The work described in this chapter has demonstrated that adenovirus-mediated overexpression of elafin in murine macrophages suppresses their inflammatory response to LPS. These data also suggest that elafin acts intracellularly to dampen macrophage responsiveness to LPS, since the anti-inflammatory effect of elafin gene augmentation appeared to be independent of elafin released extracellularly; while an anti-inflammatory effect of exogenous elafin was demonstrated in similar conditions and with comparable levels of elafin in Chapter 4, prior interaction of elafin with LPS was shown to be crucial to this result. Adenovirus-mediated elafin gene transfer may also provide cells with a negative feedback mechanism which is amplified during inflammation, since LPS was shown to upregulate elafin production by Ad-elafin-infected macrophages.

These findings suggest that adenoviral augmentation of elafin may be of benefit in inflammatory conditions such as sepsis, wherein suppression of macrophage responses to LPS is a desirable outcome, and provide further evidence that elafin may play an important role as a modulator of innate immune responses.

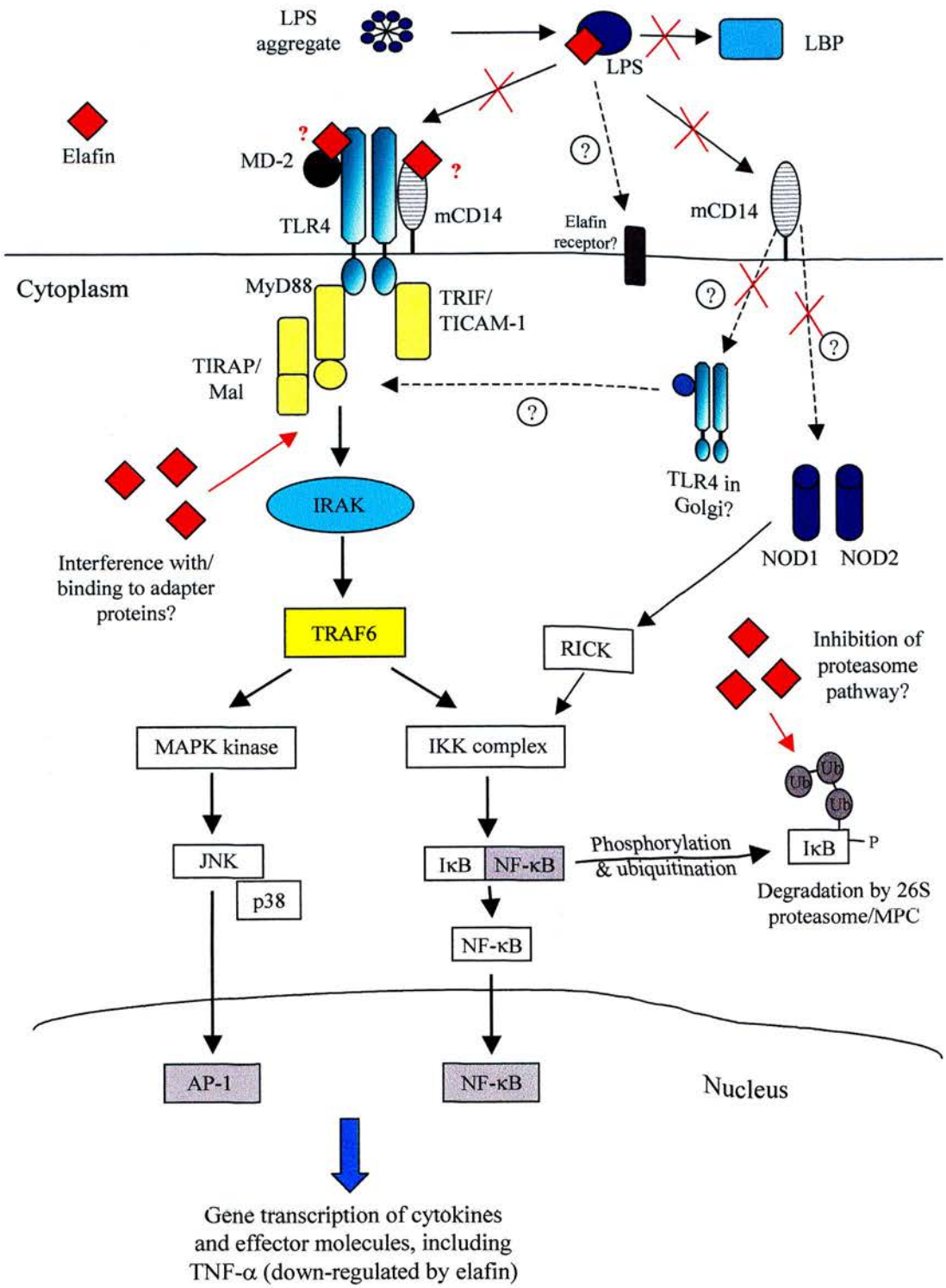
APPENDIX V

Schematic diagram of the proposed LPS signalling pathway in macrophages, leading to transcription factor activation and gene transcription (including points of potential elafin interaction)

In serum, LPS-binding protein (LBP) catalyses transfer of LPS monomers to membrane-bound CD14 (mCD14). Binding of LPS to CD14 leads to association of CD14 with the Toll-like receptor 4 (TLR4)-MD-2 complex (potential LPS recognition by intracellular TLR4 and NOD receptors is also shown). Intracellular signalling depends on interaction of the intracellular TLR domain, TIR (Toll/IL-1 receptor homology domain), with IRAK (IL-1 receptor-associated kinase); this process is facilitated by the adapter proteins, MyD88 (myeloid differentiation protein 88) and TIRAP (TIR domain-containing adapter protein, also known as MyD88 adapter-like protein or Mal). IRAK is phosphorylated and associates with the TNF-receptor-associated factor-6 (TRAF6), leading to activation of MAP kinases or the IκB kinase (IKK) complex. To induce NF-κB activation, IKKs phosphorylate IκB at serine residues, and then IκB undergoes ubiquitination at lysine residues. The polyubiquitinated IκB subunit remains complexed with NF-κB, but is then selectively degraded by the nonlysosomal, ATP-dependent 26S proteasome (multicatalytic proteinase) complex or MPC. On release, NF-κB translocates to the nucleus and induces transcriptional activation of a host of cytokines and effector molecules, including TNF-α.

Points of potential elafin inhibition are indicated on the diagram. These are discussed in the 'Discussion' section of Chapter 5. Elafin is represented by a red diamond. Question marks indicate unconfirmed steps or interactions. Red 'X' across an arrow denotes potential elafin-mediated inhibition of a step in the pathway.

Diagram and diagram description are adapted from reviews by Verma and Stevenson (1997), O'Neill (2002), Cohen (2002), Kopp and Medzhitov (2003) and Marshall (2003).


LPS aggregate
LPS
LBP
Elafin
MD-2
TLR4
mCD14
Elafin receptor?
mCD14
Cytoplasm
MyD88
TRIF/ TICAM-1
TIRAP/ Mal
IRAK
TLR4 in Golgi?
NOD1
NOD2
Interference with/ binding to adapter proteins?
TRAF6
RICK
Inhibition of proteasome pathway?
MAPK kinase
IKK complex
JNK
p38
IκB
NF-κB
Phosphorylation & ubiquitination
Ub
IκB
P
Degradation by 26S proteasome/MPC
NF-κB
AP-1
NF-κB
Nucleus
Gene transcription of cytokines and effector molecules, including TNF-α (down-regulated by elafin)

CHAPTER 6

THE EFFECT OF ELAFIN GENE AUGMENTATION ON ANTIMICROBIAL ACTIVITY OF MURINE EPITHELIAL CELLS

6.1. AIMS AND BACKGROUND

Having established in the preceding results chapters that elafin, similar to other antimicrobial peptides with which it shares several biochemical characteristics (such as low molecular weight, cationicity, heavy disulphide bonding and tissue distribution at mucosal sites), can bind to and modulate the pro-inflammatory activity of LPS, it also seemed logical to develop previous studies concerning elafin's antibacterial activity. Thus the primary aim of the work described in this chapter was to further investigate the antimicrobial properties of elafin, using the Ad-elafin vector. As discussed in Chapter 1, it has previously been demonstrated that elafin peptides possess antimicrobial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa in vitro* (Simpson *et al.*, 1999; Meyer-Hoffert *et al.*, 2003), and that adenoviral augmentation of elafin can protect the murine lung against acute injury mediated by *P. aeruginosa in vivo* (Simpson *et al.*, 2001b).

Consequently, the experiments described in this chapter were performed in order to expand and rationalise these findings by using an *ex vivo* model of airway epithelium. We wished to investigate whether adenoviral over-expression of elafin would confer to murine epithelial cells enhanced antimicrobial activity against the pulmonary pathogens *S. aureus* and *P. aeruginosa,* Gram-positive and Gram-negative organisms respectively. For this purpose, the antimicrobial activities of Ad-elafin-infected murine Clara cells (DJS2/2) and primary tracheal epithelial cells were studied. It must be noted at this point that preliminary experiments investigating the antimicrobial activity of Clara cells were carried out using only *P.aeruginosa.*

Expertise in the preparation of differentiated murine tracheal epithelium existed locally within a related research group (Davidson *et al.*, 2000); consequently,

the majority of the antimicrobial assays described in this chapter were performed in collaboration with Dr. Alison Maxwell and Dr. Julia Dorin of the MRC Human Genetics Unit, Western General Hospital, Edinburgh.

For investigation of the antimicrobial activity of Ad-elafin, the bacteria *P.aeruginosa* and *S. aureus* were selected for these studies for five main reasons. Firstly, both can be considered as versatile and dangerous pathogens in humans, in particular in the context of pulmonary infection in cystic fibrosis (CF) patients (Govan and Nelson, 1992; Hutchison and Govan, 1999), in settings of immune compromise (Maurer *et al.*, 1992; Musher *et al.*, 1994; Dropulic *et al.*, 1995) and in the pathogenesis of sepsis (Dudley, 1990; Bone, 1994). Secondly, we wished to study the activity of Ad-elafin-infected cells against both a Gram-negative (*P. aeruginosa*) and a Gram-positive (*S. aureus*) organism. Thirdly, novel strategies to treat infection caused by these organisms are desirable since they are known to harbour mechanisms which confer to them resistance to common antimicrobial agents (Hancock, 1998; Smith and Jarvis, 1999). Fourthly, tremendous experience in the biology of these organisms exists locally within a related group involved chiefly in the research of CF microbiology (Govan and Deretic, 1996). Finally, since synthetic elafin had previously been demonstrated by Simpson *et al.* (1999) to possess antimicrobial activity against both *P. aeruginosa* and *S. aureus*, it also seemed prudent to develop our understanding of Ad-elafin using these organisms. The following sections briefly discuss *P. aeruginosa* and *S. aureus*.

6.1.1. *Pseudomonas aeruginosa*

P. aeruginosa is a ubiquitous environmental non-fermentative Gram-negative bacillus, found in habitats such as soil, water, plants and vegetables (Green *et al.*, 1974; Vasil *et al.*, 1986). In the hospital setting, *P. aeruginosa* can often be cultured from sites including sinks, respirators and humidifiers, as well as from the hands of medical personnel (Pitt, 1986). Furthermore, recent findings have indicated that *P. aeruginosa* is the fourth leading cause of nosocomial infection and the leading cause of hospital-acquired pneumonia (Jarvis and Martone, 1992).

This organism is regarded as an opportunistic pathogen, since it typically causes disease only in individuals with impaired host defences and has difficulty accessing healthy tissues and adhering to host cells (Grimwood, 1992). *P. aeruginosa* can readily cause infection in patients who are generally immunosuppressed and therefore predisposed to a variety of bacterial and fungal infections, such as neutropenic patients undergoing chemotherapy or individuals suffering from AIDS (Bendig *et al.*, 1987; Franzetti *et al.*, 1992; Kielhofner *et al.*, 1992). Alternatively, infection may result when the host's defences are artificially breached as in the case of assisted ventilation or by the presence of an indwelling urinary catheter (Neu, 1983). *P. aeruginosa* is also one of the top three pathogens responsible for sepsis due to Gram-negative bacteria (along with *E. coli* and *K. pneumoniae*) (Dudley, 1990). In terms of conditions more specific to the pathogenesis of *P. aeruginosa*, this bacterium is more commonly associated with bacteraemia in severe burn victims (McManus *et al.*, 1985; Tredget *et al.*, 1992), acute ulcerative keratitis in users of extended-wear contact lenses (Alfonso *et al.*, 1986; Zhao and Panjwani, 1995), and chronic lung infection in CF patients (reviewed by Govan and Deretic, 1996). CF patients in particular are highly susceptible to pulmonary infections with *P. aeruginosa*; indeed, over 90% of CF patients are colonised by *P. aeruginosa* by the time they reach adulthood (Hutchison and Govan, 1999). Chronic infection with this organism is also the cause of death in over three-quarters of CF patients (Pier, 2002).

Several host- and bacterial-derived factors contribute to colonisation of the airways of CF patients by *P. aeruginosa*. CF is an autosomal recessive disorder resulting from mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a cyclic AMP-dependent chloride ion channel localised to the apical plasma membrane in epithelial cells (Goldberg and Pier, 2000). The increased pulmonary susceptibility of CF patients to bacterial pathogens is associated with airway surface liquid (ASL) dehydration, decreased transport velocity, mucus hypersecretion, and a concomitant deficiency in airway antimicrobial peptide activity (reviewed by Pier, 2002). Recent findings have demonstrated that CFTR itself may act as a receptor for *P. aeruginosa* and mediate

its epithelial uptake and clearance in healthy lungs (Pier *et al.*, 1996; Pier *et al*, 1997; Pier, 2000). Moreover, CFTR has been proposed as an epithelial pattern recognition molecule (PRM) responsible for the binding, extracting and endocytosis of *P. aeruginosa* LPS, leading to activation of NF-κB (Schroeder *et al.*, 2002). CF epithelial cells may also represent a more attractive substrate for *P. aeruginosa* adhesion, due to their increased surface expression of the glycolipid asialo GM_1 (Saiman and Prince, 1993).

P. aeruginosa elaborates an array of potent virulence factors which may contribute to its pathogenicity in the compromised patient. These include LPS, exotoxin A, leukocidin, exoenzyme S, bacterial elastase, phospholipase C, proteases and pigments such as pyocyanin (reviewed by Wilson and Dowling, 1998). Perhaps most strikingly, *P. aeruginosa* can convert from a non-mucoid phenotype to a mucoid phenotype, characterised by production of the exopolysaccharide alginate; this conversion to mucoidy is synonymous with chronic infection in CF following initial colonisation by non-mucoid strains, and confers to the organism enhanced antibiotic resistance and protection against host defences (Govan and Deretic, 1996). The emergence of mucoid *P. aeruginosa* is often correlated with a poor prognosis (Pedersen *et al.*, 1992; Koch and Hoiby, 1993), forming the rationale for strategies to eradicate the organism during the initial non-mucoid stages of colonisation (Valerius *et al.*, 1991; Frederiksen *et al.*, 1997).

Effective antibiotic therapy of *P. aeruginosa* is hindered by the high intrinsic resistance of this organism to antimicrobial agents (Hancock, 1998). The outer membrane of *P. aeruginosa* has a low permeability for passage of antibiotics, while the organism also employs multidrug efflux pumps and antibiotic-inactivating enzymes such as β-lactamases (Hancock and Woodruff, 1988; Poole *et al.*, 1993; Poole *et al.*, 1996; Philippon *et al.*, 1997; Hancock, 1998); the combination of these factors renders treatment of *P. aeruginosa* extremely difficult.

Two strains of *P. aeruginosa* were used in this work, PAO1 and J1385, although the majority of experiments were performed using PAO1; both of these

strains are non-mucoid. PAO1 is the best characterised of all laboratory strains, and indeed its complete genome sequence has recently been characterised (Stover *et al.*, 2000). This strain has been demonstrated to generate pulmonary pathology in rodent models (George *et al.*, 1993). *P. aeruginosa* J1385 is a CF clinical isolate.

6.1.2. *Staphylococccus aureus*

S. aureus is a Gram-positive coccus with a characteristic propensity to arrange into clusters, for which humans may be considered a natural reservoir; *S. aureus* is found often as part of the normal microflora of human skin, the upper respiratory tract and the intestinal tract (Bachert *et al.*, 2002). Between 30-50% of healthy adults are colonised, with 10-20% persistently colonised, and those persons colonised with *S. aureus* are at increased risk for subsequent infections (Casewell and Hill, 1986; Wenzel and Perl, 1995). Staphylococcal colonisation rates are high among patients with type 1 diabetes, intravenous drug users, individuals undergoing haemodialysis, surgical patients and patients with AIDS (Tuazon and Sheagren, 1974; Tuazon *et al.*, 1975; Yu *et al.*, 1986; Weinke *et al.*, 1992; Kluytmans *et al.*, 1995).

Although *S. aureus* is a commensal organism, it can also be considered as a major pathogen with regard to both community-acquired and nosocomial infection. *S. aureus* is a prominent causative agent of skin, soft tissue, respiratory, bone, joint and endovascular disorders (Lowy, 1998), and the majority of these infections occur in those with multiple risk factors for infection (Musher *et al.*, 1994). The typical pathological finding of staphylococcal disease is abscess formation, since leukocytes are the primary host defence against *S. aureus* infection (Verdrengh and Tarkowski, 1997).

A minority of bacteraemic or local *S. aureus* infections can lead to sepsis, and these cases are usually associated with risk factors such as immunosuppression, chemotherapy or invasive procedures (Lowy, 1998). Pathophysiological effects of staphylococcal sepsis are similar to those of Gram-negative sepsis, with fever, hypotension and tachycardia in most cases, and progression to multiple organ failure,

disseminated intravascular coagulation and death in severe cases (Lowy, 1998). *S. aureus* is one of the most prevalent Gram-positive organisms in cases of sepsis (Bone, 1994).

S. aureus is also closely associated with pulmonary disease in CF (Govan and Nelson, 1992). *S. aureus* typically infects the lungs of CF patients in infancy, prior to colonisation of lungs by *P. aeruginosa* in adolescence and adulthood (Govan and Deretic, 1996). Pulmonary infections with *S. aureus* can usually be controlled with aggressive antibiotic therapy, but effective clearance of these organisms allows subsequent colonisation and chronic establishment of *P. aeruginosa* (Govan and Deretic, 1996).

This organism exhibits a plethora of potent virulence factors, such as peptidoglycan, enterotoxins, toxic shock syndrome toxin 1(TSST-1), proteases and hyaluronidase (reviewed by Lowy, 1998). Additionally, the increasing association of *S. aureus* with antimicrobial resistance is a major concern for clinicians. While expression of β-lactamase by *S. aureus* confers to the organism resistance against penicillin, the presence of the *mec* gene can confer resistance to practically all β-lactam antibiotics (reviewed by Hiramatsu *et al.*, 2001). Such organisms are known as methicillin-resistant *S. aureus* (MRSA), and are profoundly refractory to treatment with conventional antibiotics. Indeed, the recent emergence of vancomycin-resistant MRSA poses a major therapeutic challenge (Hiramatsu *et al.*, 1997a; Hiramatsu *et al.*, 1997b).

The strain used in this study, *S. aureus* C1705, is a clinical isolate from a patient with CF.

6.2. RESULTS

6.2.1. The effects of elafin gene augmentation on the antimicrobial activity of Clara cells against *P. aeruginosa*

It was hypothesised that overexpression of elafin, which in synthetic peptide form has previously been shown to inhibit bacterial growth *in vitro*, could enhance the antibacterial activity of airway epithelial cells against the common pathogens *P. aeruginosa* and *S. aureus*. Initial experiments designed to investigate the effects of Ad-elafin infection on the antimicrobial activity of epithelial cells used the murine Clara cell line DJS2/2, described previously in Chapter 4, and assessed only activity against the *P. aeruginosa* strain PAO1. These experiments were conducted using conventional tissue culture conditions as described in the legend below Figure 1. Several difficulties were encountered in the execution of these assays, and subsequently the reproducibility of these experiments was poor. For example, optimisation of the parameters for these investigations was hampered by a profound inconsistency in initial bacterial numbers; attempts were made to standardise these counts by measuring the absorbance of bacterial cultures, but this technique itself was found to yield hyper-variable results. Moreover, numerous conflicting results were obtained between assays and this hindered attempts to draw any solid conclusions concerning the effects of Ad-elafin infection.

We initially postulated that infecting Clara cells would enhance their antimicrobial activity against PAO1, on account of the previous demonstration that elafin has inherent antimicrobial activity against this organism (Simpson *et al.*, 1999). On a few occasions Ad-elafin-infected cells seemed to display an enhanced antimicrobial activity, but these findings were mirrored equally by experiments that suggested no significant killing of bacteria. Thus, we originally surmised that the assay conditions may not have been optimal for investigating the antimicrobial activity of elafin. For instance, elafin secreted by the cells may have been too dilute to exert an antimicrobial effect; equally, the bacterial numbers may have been too great and so overwhelming the exogenous elafin. Furthermore, the antimicrobial activity displayed by several similar peptides such as human β-defensins 1 and 2 has

been shown to be salt-sensitive (Goldman *et al.*, 1997; Bals *et al.*, 1998b). The salt-sensitivity of elafin's antimicrobial activity has not been investigated to date, but the salt concentrations in tissue culture conditions used here may have potentially affected bacterial killing by elafin. However, the salt concentrations of media were not measured during these experiments.

To further our understanding of the lack of an observed elafin antimicrobial activity against PAO1, Western blots were carried out to analyse the elafin content of medium derived from antimicrobial assays (Figure 1). Firstly, the concentration of elafin secreted by Ad-elafin-infected cells was shown to increase in a time-dependent manner post-viral infection (lanes 1 and 2). No elafin was detected in medium taken from cells that had not been infected with Ad-elafin (lane 4). Incubation of non-virally-infected cells with PAO1 was associated with the detection of an extra band (demonstrated by an arrow labelled 'PAO1 band'), and this band was shown to be specific to the presence of bacteria in culture medium since it was present in lane 5 but not in lane 4. This band likely represents non-specific antibody interactions with a bacterial product. Most interestingly, on comparison of media taken from Ad-elafin-infected cells incubated either in the presence or absence of bacteria, it was noted that elafin could not be detected in the media of cells that had been exposed to PAO1 (comparing lanes 2 and 3). These findings were supported by analysis of cell supernatants by elafin ELISA (not shown). The observation that the 'PAO1 band' was present when both Ad-elafin-infected and non-virally-infected cells were incubated with bacteria rules out the possibility that the band in lane 3 could be due, for example, to detection of elafin bound to a higher molecular weight protein derived from PAO1. No other bands were detected on analysis of media taken from Ad-elafin cells incubated with PAO1. Thus we hypothesised that the lack of an observed antimicrobial effect, in assays utilising Ad-elafin-infected Clara cells *in vitro*, may be attributed to the potential metabolism or destruction of elafin by PAO1.

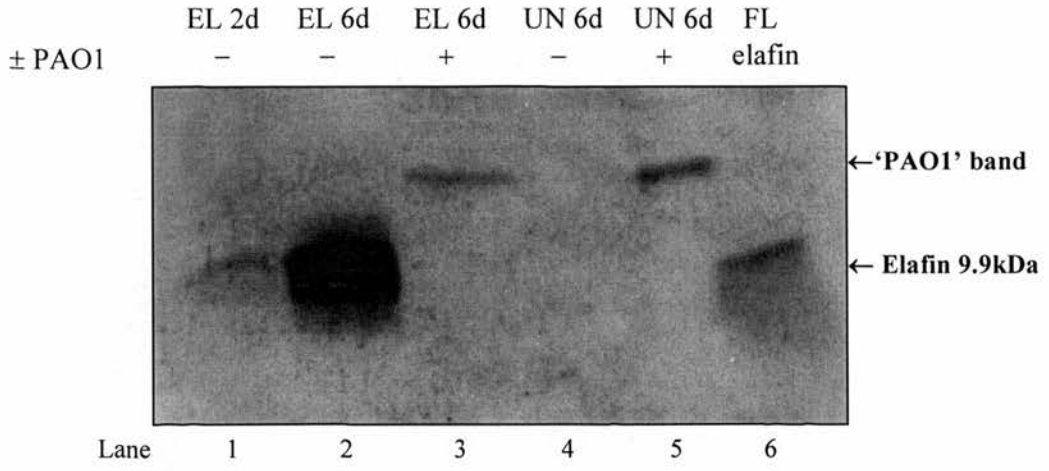


Figure 1. Elafin secreted by Ad-elafin-infected murine Clara cells is eradicated by *Pseudomonas aeruginosa* PAO1

Murine Clara cells of the line DJS2-2 were grown to confluence in 96-well plates, infected with Ad-elafin (moi 100) or vehicle alone and incubated for 2-6 days at 37°C in 100μl DMEM lacking penicillin-streptomycin. 100μl samples of medium or medium containing PAO1 were added and incubated with cells for 5 hours at 37°C. Media were UV-inactivated and subjected to SDS-PAGE/ western blotting using anti-elafin polyclonal antibody. 2d = medium from cells 2 days post-viral infection, 6d = medium from cells 6 days post-viral infection. + = PAO1 added to cells, – = medium only added to cells. EL = cells infected with Ad-elafin, UN = cells uninfected with virus. 40ng of synthetic full-length elafin was used as a control (lane 6).

6.2.2. Adenoviral infection of primary murine tracheal epithelium

In order to advance our understanding of the effects of elafin on the growth and survival of bacteria, we resolved to use a cell system that eliminated the opacity intrinsic to the aforementioned tissue culture conditions. Accordingly, primary culture models of murine tracheal epithelium were generated and grown on semi-permeable membranes at an air interface, according to a technique developed by Davidson *et al.* (2000), and these cultures were used to further investigate the potential antimicrobial activity conferred by Ad-elafin.

In the previous section, viral infections of Clara cells were carried out exclusively in the normal DMEM in which these cells are routinely cultured. It must be noted that, in chronological terms, our laboratory group became aware of the benefit of CaPi precipitates in enhancing viral infection efficiency subsequent to completion of the above experiments. Moreover, the use of techniques to augment adenoviral infection had been deemed unnecessary due to the presence of the Coxsackie/adenovirus receptor in lung epithelium (Tomko *et al.*, 1997). However, several studies have reported poor adenoviral gene transfer to airway epithelia due to low apical receptor expression, and an improvement in infection efficiency using CaPi precipitates (Zabner *et al.*, 1997; Fasbender *et al.*, 1998). Hence, following the success of utilising CaPi precipitates in infection medium for the macrophage experiments described in Chapter 5, we decided to investigate whether this technique would also enhance adenoviral infection of primary tracheal epithelium. Using Ad-lacZ as a reporter construct, infection of tracheal epithelial cells was shown to be ameliorated by adding virus in an infection medium comprising MEEM plus 4mM Ca^{2+} (generating CaPi precipitates formed with a final concentration of 5.8mM Ca^{2+} and 0.86mM Pi, as described in Chapter 5) (Figure 2). Infection efficiency was satisfactory when virus was incubated with cells in the same type of medium used to feed the cells from beneath the semi-permeable support membrane, Ultroser G (USG) medium (Panel C). However, infection efficiency was enhanced 2-3-fold by CaPi (Panel D), and therefore this infection medium was utilised for all subsequent experiments.

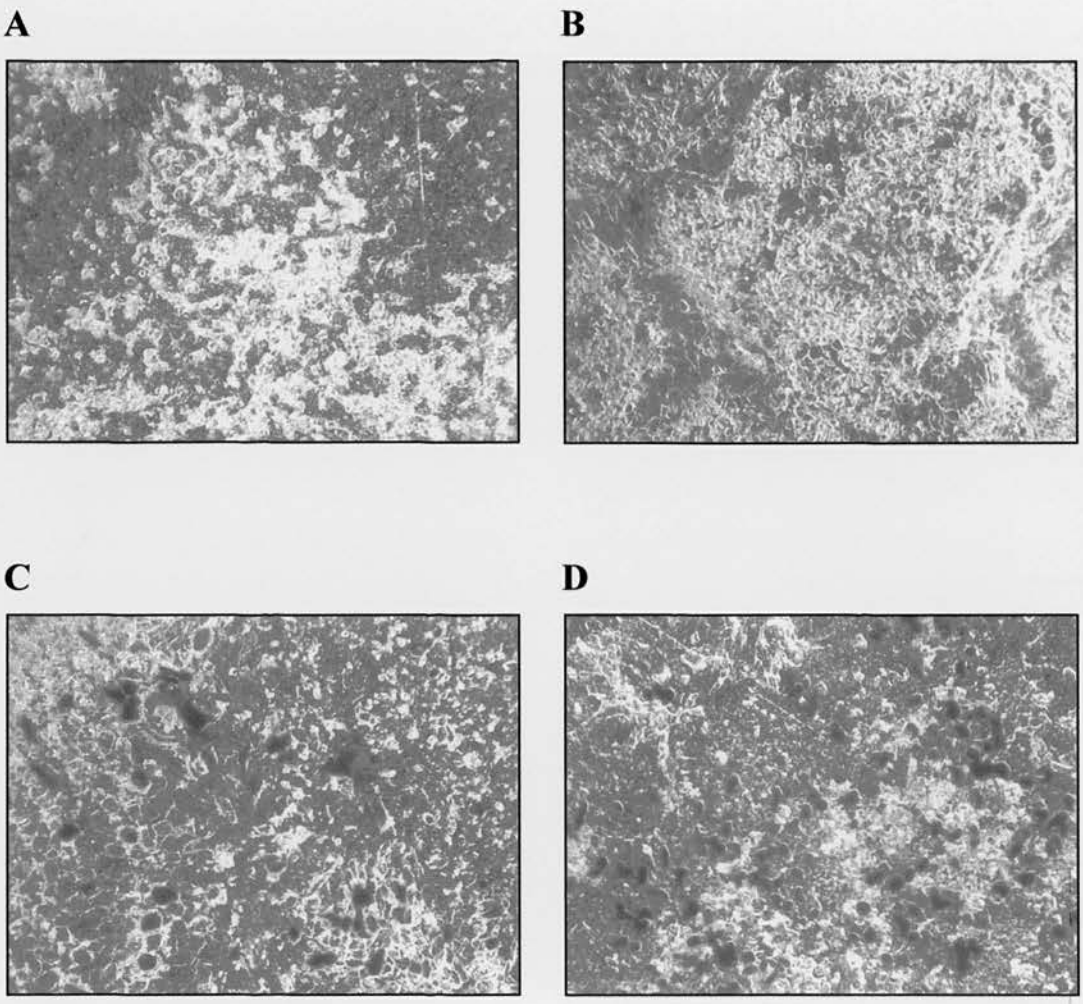


Figure 2. Adenovirus efficiently infects primary murine tracheal epithelial cells and infection is enhanced by calcium phosphate (CaPi) precipitates

Primary tracheal epithelial cells derived from C57BL/6J were treated with vehicle alone or Ad-lacZ at moi 100, and incubated for 18-20 hours at 37°C. Cells were then subjected to X-gal staining. Blue colouration indicates expression of the β-galactosidase gene.

PANEL A - no viral infection of cells, incubated in USG medium
PANEL B - no viral infection of cells, incubated in MEEM plus 4mM Ca^{2+}
PANEL C – cells infected with Ad-lacZ (moi 100) in USG medium
PANEL D – cells infected with Ad-lacZ (moi 100) in MEEM plus 4mM Ca^{2+}

USG medium = Ultroser G medium; MEEM = Minimum Essential Eagle's Medium

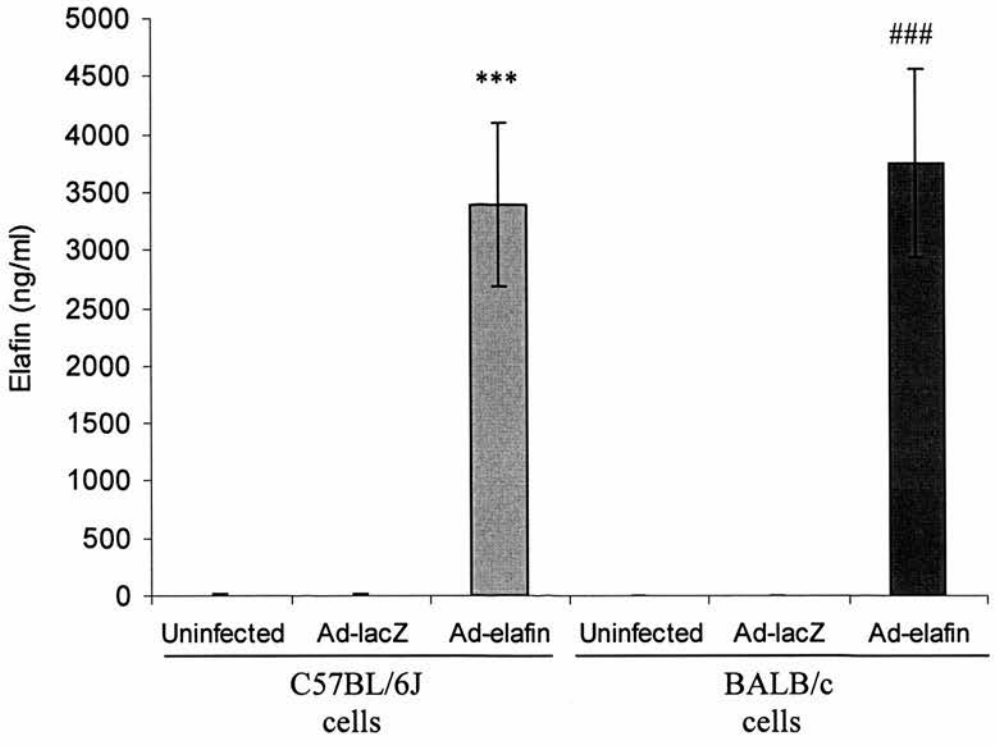


Figure 3. Ad-elafin-infected primary murine tracheal epithelial cells secrete high levels of elafin

Primary tracheal epithelial cells derived from C57BL/6J or BALB/c mice were infected with Ad-elafin or Ad-lacZ (moi 100 for each) or vehicle alone and incubated for 18-20 hours at 37°C. Cell inserts were then washed with both 105µl phosphate buffer (0.008M K_2HPO_4/ 0.002M KH_2PO_4, pH 7.4) and 105µl phosphate buffer/0.5% Triton-X. These two samples were then pooled and analysed by elafin ELISA (see 'Materials and Methods'). Values represent either mean ± SD of n=3 experiments (C57Bl/6J) or n=1 experiment (BALB/c), each performed in triplicate. For C57BL/6J cells, *** = significant difference, $P<0.001$, compared with uninfected or Ad-lacZ-infected cells. For BALB/c cells, ### = significant difference, $P<0.001$, compared with uninfected or Ad-lacZ-infected cells.

Furthermore, considerable levels of elafin were secreted by Ad-elafin-infected cells (Figure 3). Cells derived from two different mouse strains, C57Bl/6J and BALB/c, both secreted similar concentrations of elafin; neither of these cell types secreted a significant level of protein detected by the anti-elafin antibody unless infected with Ad-elafin (Figure 3), confirming previous observations in our laboratory that the anti-human elafin antibody does not cross-react with any murine protein.

6.2.3. The effects of elafin gene augmentation on the antimicrobial activity of primary murine tracheal epithelium against *P. aeruginosa*

It should be noted prior to describing these experiments that, while two strains of mice were used initially to generate primary culture models, the majority of experiments described herein were performed using cells derived from C57BL/6J mice. The decision to persist with only one mouse strain was made due to restrictions on both time and cost, while C57BL/6 mice were selected since cells derived from this strain were characterised in the original paper by Davidson *et al.* (2000). Moreover, this strain has previously been used in-house to generate both elafin transgenic (Sallenave *et al.*, 2003) and murine β defensin 1-deficient mice (Morrison *et al.*, 2002a). Antimicrobial assays involving the *P. aeruginosa* strain PAO1 were carried out exclusively using C57BL/6J cells, whereas those involving the strain J1385 were performed using both C57BL/6J and BALB/c cells.

The first interesting observation to be drawn from these data is that untreated tracheal cells were capable of killing PAO1, i.e. following 3-hour incubation with cells the numbers of PAO1 colonies recovered were lower than those added at the outset (Figure 4). C57Bl/6 mice have also been shown to effectively clear PAO1 following lung instillation *in vivo* (Cressman *et al.*, 1998). In order to demonstrate this phenomenon, and to illustrate a typical series of results provided by counting colony forming units (cfu) of PAO1, Figure 4 shows the results of a single experiment. Figure 5 shows the combined data from two separate experiments, with cfu values expressed as a percentage of colony growth on uninfected cells. It should

be noted that Triton-X was incorporated into the second phosphate buffer wash to ensure removal of bacteria still adhered to the cell surface, following the first wash.

Ad-elafin infection of epithelium resulted in significantly enhanced survival and growth of PAO1 (Figures 4 and 5). In comparison with uninfected cells, PAO1 numbers were increased by approximately 440% on incubation with Ad-elafin-infected cells (Figure 5). Infection of cells with Ad-lacZ resulted in a notable but non-significant compromise of the ability of tracheal epithelium to kill PAO1 (Figures 4 and 5).

In order to investigate whether the facilitation of enhanced survival of PAO1 by Ad-elafin was specific to this strain of *P. aeruginosa*, we also examined the effects of Ad-elafin on the clinical strain J1385. Although this investigation only encompassed a single experiment (carried out in triplicate), a similar trend was observed to that obtained with PAO1 (Figure 6). Using cells derived from both mouse strains, Ad-elafin resulted in an unequivocal augmentation of survival of J1385 and this was significant compared with bacterial growth on either uninfected or Ad-lacZ-infected cells (Figure 6). Uninfected BALB/c cells appeared to have superior ability to kill J1385 (compared with the killing elicited by uninfected C57BL/6J cells) and, accordingly, the growth of J1385 on Ad-elafin-infected BALB/c cells was not as pronounced.

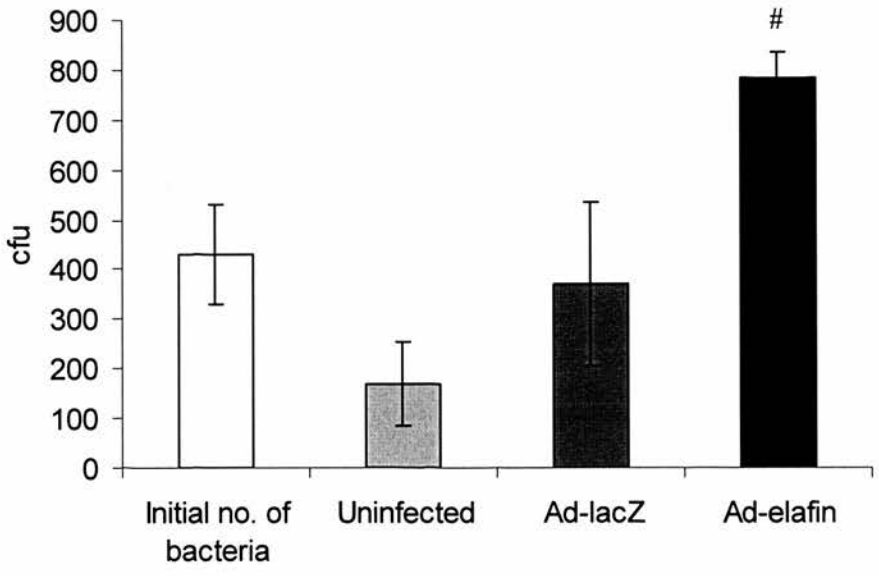


Figure 4. Ad-elafin infection of primary tracheal epithelial cells derived from C57Bl/6J mice compromises their ability to kill *Pseudomonas aeruginosa* PAO1, and enhances bacterial survival

Primary tracheal epithelial cells derived from C57BL/6J mice were infected with Ad-elafin or Ad-lacZ (moi 100 for each) or vehicle alone and incubated for 18-20 hours at 37°C. 20 nanolitres (nl) of a suspension of PAO1 was added to cells and incubated for 3 hours at 37°C. Cell inserts were washed with both 105μl phosphate buffer and 105μl phosphate buffer/0.5% Triton-X, and washes plated on PIA. Agar plates were incubated overnight at 37°C prior to colony counting. Representative data from one of three similar experiments are shown. Values represent mean ± S.E., carried out in triplicate except 'Initial no. of bacteria' where twelve replicates were performed. # = significant difference, $P<0.05$, compared with uninfected cells.

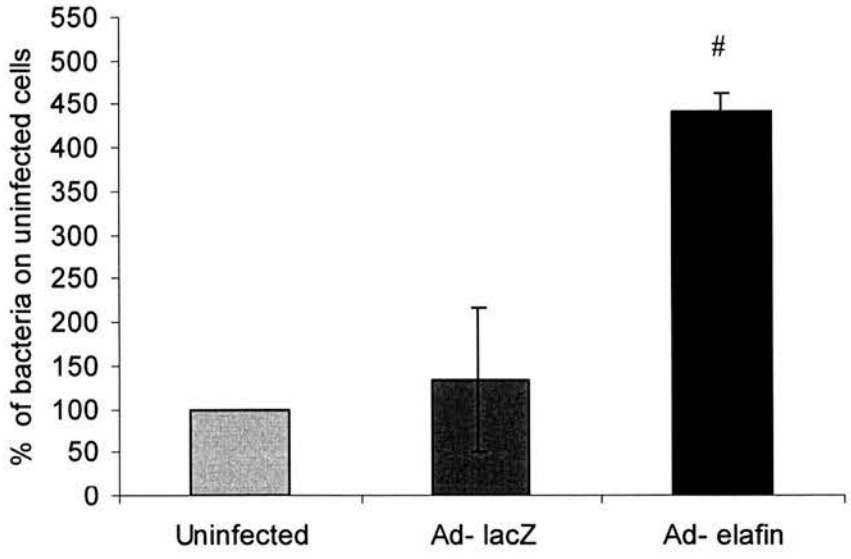


Figure 5. Ad-elafin-infected primary tracheal epithelial cells, derived from C57Bl/6J mice, significantly enhance survival of *Pseudomonas aeruginosa* PAO1, compared with uninfected or Ad-lacZ-infected cells

Primary tracheal epithelial cells derived from C57BL/6J mice were infected with Ad-elafin or Ad-lacZ (moi 100 for each) or vehicle alone and incubated for 18-20 hours at 37°C. 20 nanolitres (nl) of a suspension of PAO1 was added to cells and incubated for 3 hours at 37°C. Cell inserts were washed with both 105μl phosphate buffer and 105μl phosphate buffer/0.5% Triton-X, and washes plated on PIA. Agar plates were incubated overnight at 37°C prior to colony counting. Values represent mean ± S.E. of n=2 experiments, each performed in triplicate. # = significant difference, $P<0.05$, compared with uninfected cells.

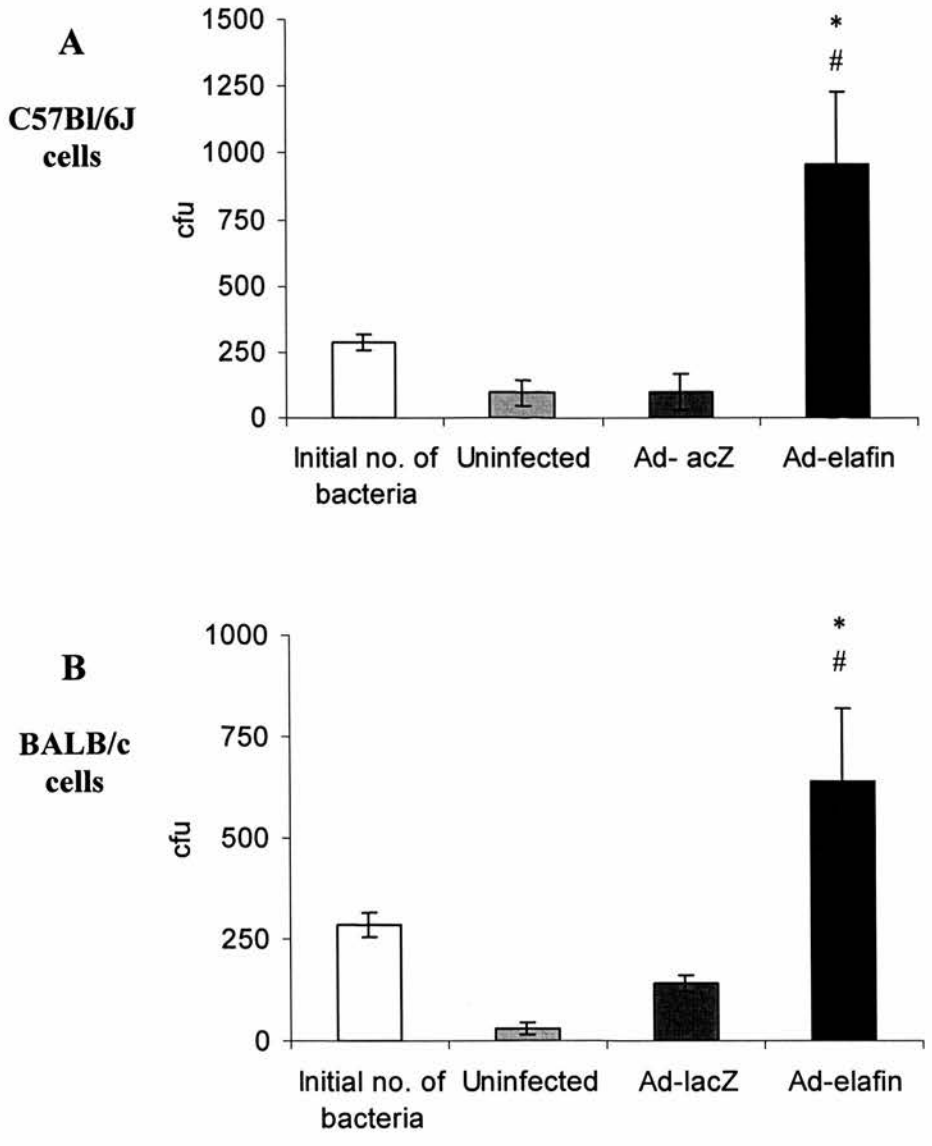


Figure 6. Ad-elafin infection of primary tracheal epithelial cells derived from both C57Bl/6J and BALB/c mice compromises their ability to kill *Pseudomonas aeruginosa* J1385, and enhances bacterial survival

Primary tracheal epithelial cells derived from either mouse strain were infected with Ad-elafin or Ad-lacZ (moi 100 for each) or vehicle alone and incubated for 18-20 hours at 37°C. 20 nanolitres (nl) of a suspension of J1385 was added to cells and incubated for 3 hours at 37°C. Cell inserts were washed with both 105µl phosphate buffer and 105µl phosphate buffer/0.5% Triton-X, and washes plated on PIA. Agar plates were incubated overnight at 37°C prior to colony counting. Data are representative of a single experiment. GRAPH A – cells derived from C57Bl/6 mice. GRAPH B – cells derived from BALB/c mice. Values represent mean ± S.E., carried out in triplicate except 'Initial no. of bacteria' where twelve replicates were performed. * = significant difference, $P<0.05$, compared with Ad-lacZ-infected cells. # = significant difference, $P<0.05$, compared with uninfected cells.

6.2.4. The effects of elafin gene augmentation on the antimicrobial activity of primary murine tracheal epithelium against *S. aureus*

Taking into account the previous demonstration by Simpson *et al.* (1999) that synthetic elafin peptides possess antimicrobial activity against *S. aureus* C1705, it seemed appropriate to investigate whether Ad-elafin infection of tracheal epithelium would enhance killing of this organism.

Figure 7 shows the results of a single experiment using cells derived from C57BL/6J mice, with data provided as colony counts of *S. aureus*. In contrast to the observed antimicrobial activity of epithelium against PAO1 (Figure 4), untreated tracheal epithelial cells were unable to prevent growth of C1705 (Figure 7). Most notably, Ad-elafin infection of C57 BL/6J tracheal epithelium resulted in enhanced antimicrobial activity against C1705, and this effect was significant over three experiments (Figure 7 and Figure 8); compared with uninfected cells, Ad-elafin-infected cells reduced bacterial numbers by an average of 63% (Figure 8). Likewise, Ad-elafin infection of tracheal epithelium derived from BALB/c mice resulted in recovery of a significantly decreased number of colonies of C1705 (Figure 9). In the case of BALB/c cells, the effects of Ad-elafin were not as pronounced, but still resulted in an approximately 30% reduction in survival of C1705 (compared with uninfected cells). It is worthy to note that the enhanced antimicrobial activity conferred by Ad-elafin was insufficient to reduce bacterial numbers below those added to the cells at the outset, as demonstrated using C57BL/6J cells (Figure 7). A further interesting observation was that, in several assays, infection of cells with Ad-lacZ augmented their antimicrobial activity (Figure 7 and Figure 9).

Finally, analyses of the phosphate buffer used to collect bacteria from the surface of Ad-elafin-infected tracheal epithelium demonstrated that incubation of C1705 with these cells caused a significant up-regulation in the level of elafin secreted (Figure 10). Regrettably, similar data regarding elafin levels secreted by cells following incubation with PAO1 are unavailable, as a result of alterations to the assay protocol as these experiments progressed.

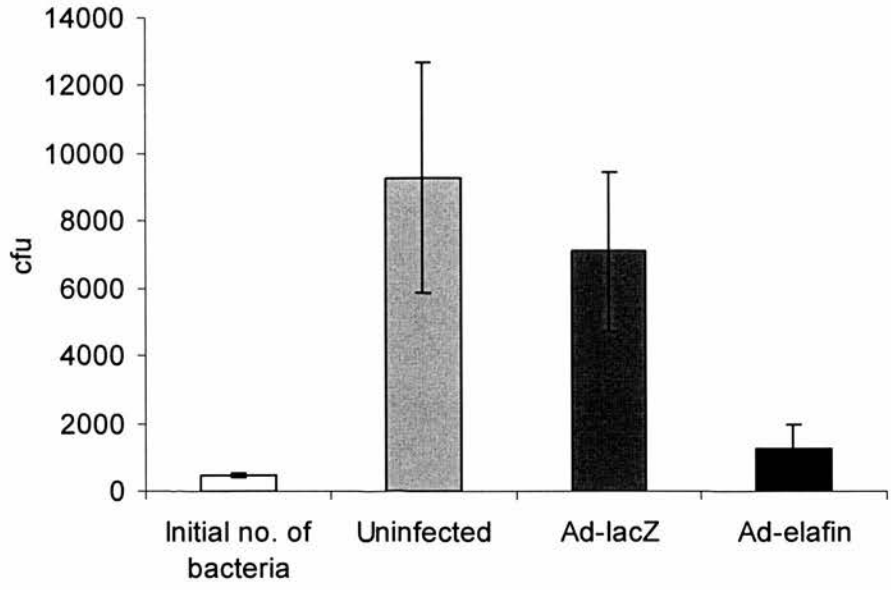


Figure 7. Ad-elafin infection of primary tracheal epithelial cells derived from C57Bl/6J mice enhances their antimicrobial activity against *Staphylococcus aureus* C1705

Primary tracheal epithelial cells derived from C57BL/6J mice were infected with Ad-elafin or Ad-lacZ (moi 100 for each) or vehicle alone and incubated for 18-20 hours at 37°C. 20 nanolitres (nl) of a suspension of C1705 was added to cells and incubated for 3 hours at 37°C. Cell inserts were washed with both 105µl phosphate buffer and 105µl phosphate buffer/0.5% Triton-X, and washes plated on nutrient agar. Agar plates were incubated overnight at 37°C prior to colony counting. Representative data from one of three similar experiments are shown. Values represent mean ± S.E., carried out in triplicate except 'Initial no. of bacteria' where twelve replicates were performed.

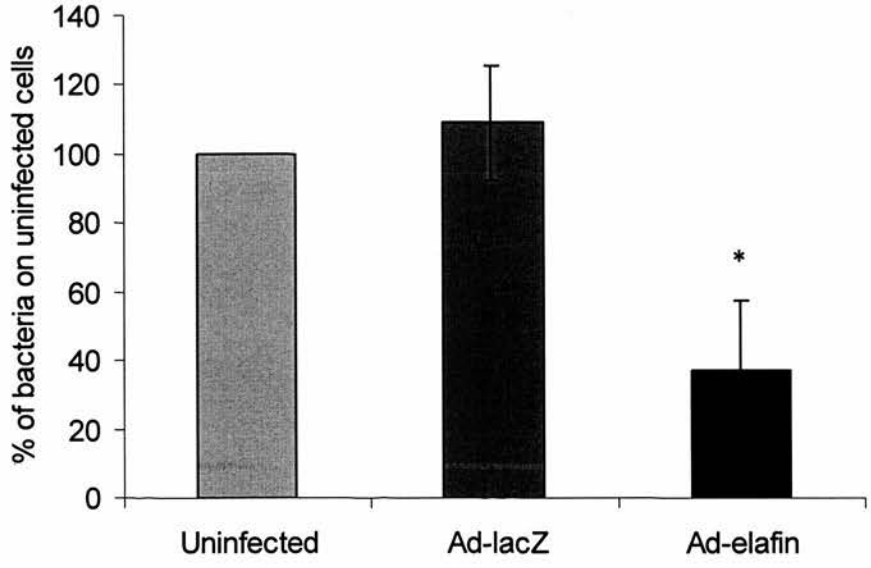


Figure 8. Ad-elafin-infected primary tracheal epithelial cells, derived from C57Bl/6J mice, exhibit significantly greater antimicrobial activity against *Staphylococcus aureus* C1705 than Ad-lacZ-infected cells

Primary tracheal epithelial cells derived from C57BL/6J mice were infected with Ad-elafin or Ad-lacZ (moi 100 for each) or vehicle alone and incubated for 18-20 hours at 37°C. 20 nanolitres (nl) of a suspension of C1705 was added to cells and incubated for 3 hours at 37°C. Cell inserts were washed with both 105µl phosphate buffer and 105µl phosphate buffer/0.5% Triton-X, and washes plated on nutrient agar. Agar plates were incubated overnight at 37°C prior to colony counting. Values represent mean ± S.E. of n=3 experiments, each performed in triplicate. * = significant difference, $P<0.05$, compared with Ad-lacZ-infected cells.

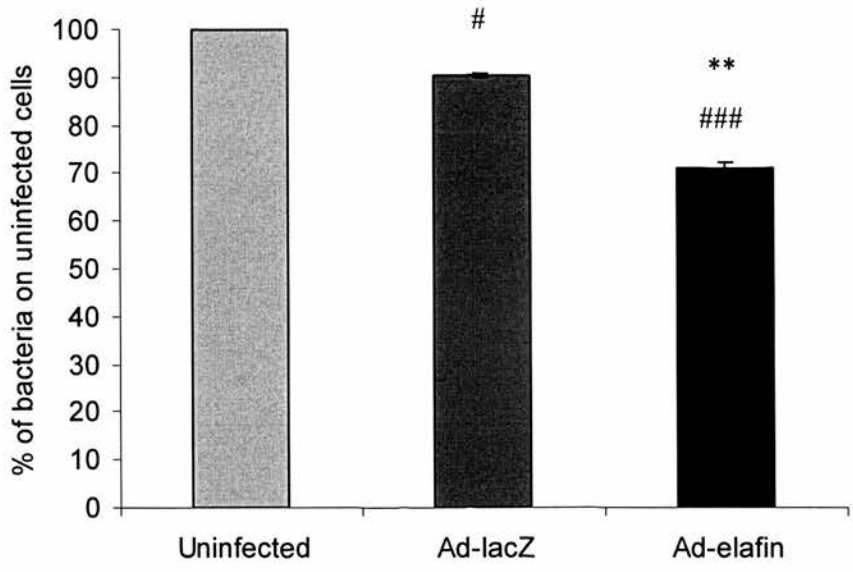


Figure 9. Ad-elafin-infected primary tracheal epithelial cells, derived from BALB/c mice, exhibit significantly greater antimicrobial activity against *Staphylococcus aureus* C1705 than either uninfected or Ad-lacZ-infected cells

Primary tracheal epithelial cells derived from BALB/c mice were infected with Ad-elafin or Ad-lacZ (moi 100 for each) or vehicle alone and incubated for 18-20 hours at 37°C. 20 nanolitres (nl) of a suspension of C1705 was added to cells and incubated for 3 hours at 37°C. Cell inserts were washed with both 105µl phosphate buffer and 105µl phosphate buffer/0.5% Triton-X, and washes plated on nutrient agar. Agar plates were incubated overnight at 37°C prior to colony counting. Values represent mean ± S.E. of n=2 experiments, both performed in triplicate. # = significant difference, $P<0.05$, compared with uninfected cells. ** = significant difference, $P<0.01$, compared with Ad-lacZ-infected cells. ### = significant difference, $P<0.001$, compared with uninfected cells.

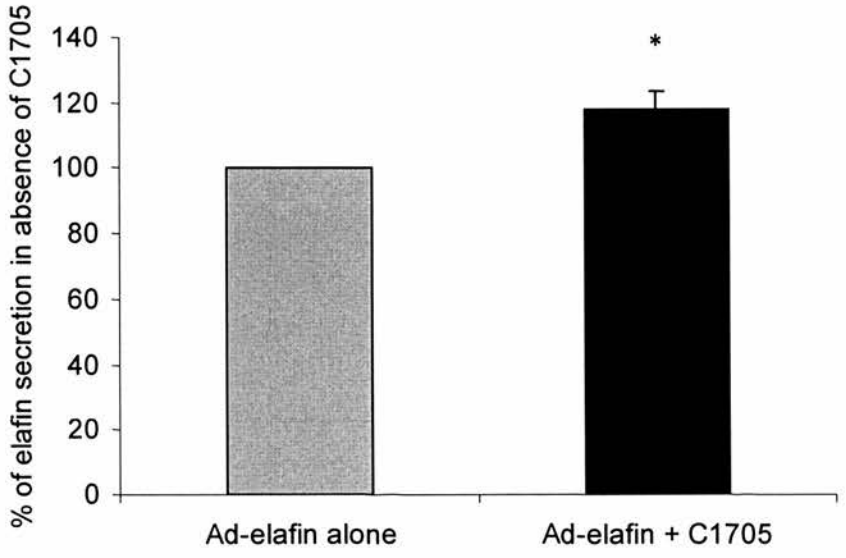


Figure 10. *Staphylococcus aureus* C1705 up-regulates elafin secretion from Ad-elafin-infected tracheal epithelial cells

Primary tracheal epithelial cells derived from C57Bl/6J mice were infected with Ad-elafin and incubated for 18-20 hours at 37°C. 20 nanolitres (nl) of a suspension of C1705 was added to cells and incubated for 3 hours at 37°C. Alternatively, cells were incubated with 20nl of phosphate buffer. Cell inserts were washed with both 105µl phosphate buffer and 105µl phosphate buffer/0.5% Triton-X, samples pooled and centrifuged at 8000rpm for 10 mins. Pellets were resuspended in 200µl phosphate buffer and plated onto nutrient agar for colony counting, while supernatants were analysed by elafin ELISA (see 'Materials and Methods'). Values represent mean ± S.E. of n=3 experiments, each performed in triplicate. * = significant difference, $P<0.05$, compared with 'Ad-elafin alone'.

6.3. DISCUSSION

The principal conclusion arising from these data is that Ad-elafin infection of primary murine tracheal epithelium modulates the antimicrobial activity of these cells against *P. aeruginosa* and *S. aureus*. Interestingly, while infection of epithelial cells with Ad-elafin significantly enhanced their antimicrobial activity against *S. aureus* (Figures 7-9), the inherent ability of cells to kill *P. aeruginosa* was greatly compromised by Ad-elafin infection and this treatment in fact augmented bacterial survival (Figures 4-6).

The primary culture model of differentiated murine tracheal epithelium described in this chapter was chosen for use in these studies for a number of reasons. Firstly, we deemed the murine tracheal epithelium to be an appropriate physiological model for bacterial invasion of the airways, in particular since both *P. aeruginosa* and *S. aureus* are known to cause infection of the trachea *in vivo* (Vishwanath and Ramphal, 1984; Baltimore *et al.*, 1989; Hutchison and Govan, 1999; Kobashi and Matsushima, 2003); moreover, tracheal epithelium is known to be an important site for initial colonisation of the upper respiratory tract of CF patients by non-mucoid *P. aeruginosa* (Fegan *et al.*, 1990; Grimwood, 1992). Secondly, since these culture models are grown on semipermeable membranes at an air-liquid interface, no culture medium bathes the cells apically; these cells thus form a confluent, polarised, ciliated epithelia, lined only by a small volume of secreted airway surface liquid as observed *in vivo* (Davidson *et al.*, 2000). For our purposes, this meant that not only could we more accurately reproduce the *in vivo* situation, but also that we could eliminate the difficulties we had experienced in performing antimicrobial assays using more conventional tissue culture conditions, in which rich media considerably augments bacterial growth (as discussed in section 6.2.1.). Finally, with regards to the potential use of adenoviral gene transfer techniques to modulate lung defence mechanisms, the tracheal epithelium may be considered as a fundamental target in the proximal airways.

Furthermore, we considered murine cells as an ideal choice for these gene transfer studies since no murine homologue of elafin has been identified to date, and our anti-elafin antibody does not appear to cross-react with any murine protein; thus, the introduction of this entirely foreign gene could allow us to study the effects of elafin without the contribution of a homologous native molecule. However, although no expression of elafin has been demonstrated in mice, elafin has previously been localised in the trachea of other species, including man (Nara *et al.*, 1994; Suzuki *et al.*, 2000).

Discussion of the results of these antimicrobial assays will be divided into separate sections for each organism, with slightly more emphasis on *P. aeruginosa* since the results obtained with this bacterium necessitate more detailed analyses.

6.3.1. The effects of elafin gene augmentation on the antimicrobial activity of primary murine tracheal epithelium against *P. aeruginosa*

The experiments using primary murine tracheal epithelium were prompted by an inability to gain consistent or informative results regarding the antimicrobial activity of adenovirally-infected Clara cells against PAO1, using standard tissue culture techniques. Nevertheless, an interesting observation arising from these initial Clara cell assays was that *P. aeruginosa* PAO1 appeared to degrade or metabolise elafin protein secreted by Ad-elafin-infected cells (Figure 1). Thus, at this point we postulated that the lack of an observed Ad-elafin-mediated antimicrobial effect may be attributable to inactivation of elafin by the bacteria, thus masking any underlying microbicidal action.

These findings were corroborated by the remarkable finding that, not only did Ad-elafin infection of tracheal epithelium compromise the inherent ability of cells to exert a bactericidal effect against PAO1, this treatment in fact significantly enhanced bacterial survival (Figures 4 and 5). We also demonstrated that this phenomenon was not restricted to the *P. aeruginosa* strain PAO1, since the clinical *P. aeruginosa* strain J1385 also exhibited improved subsistence on Ad-elafin-infected cells (Figure 6). This augmentation of *P. aeruginosa* survival was certainly associated with

secretion of high concentrations of elafin into the airway surface liquid of Ad-elafin-infected tracheal epithelium, and the complete absence of elafin secretion by uninfected or Ad-lacZ-infected cells (again confirming no cross-reactivity of the anti-human elafin antibody with a similar murine molecule) (Figure 3). It should be noted that figure 3 shows elafin levels secreted by Ad-elafin-infected epithelium following incubation without *P. aeruginosa*; unfortunately, no reliable ELISA data were obtained during these experiments regarding elafin levels following incubation with this organism.

Although both Simpson *et al.* (1999) and Meyer-Hoffert *et al.* (2003) have previously demonstrated that elafin peptides display antibacterial activity against *P. aeruginosa*, these studies produced slightly differing results. While the strains of *P. aeruginosa* used by the two groups were different (PAO1 in the study carried out by Simpson *et al.*, three ATCC strains in the study carried out by Meyer-Hoffert *et al.*), potentially contributing to discrepancies between the results, the concentrations of elafin tested in both investigations were in a similar micromolar range. The more recent study described by Meyer-Hoffert *et al.* (2003) demonstrated that elafin's antimicrobial acitivity against all three *P. aeruginosa* strains was dose-responsive, and was therefore maximal at the highest elafin concentration tested. Contrastingly, the study carried out by Simpson *et al.* (1999) demonstrated an inverse dose-response relationship for elafin's antimicrobial activity against PAO1, in that elafin's effects were maximal at a low intermediate concentration in the dose range and considerably reduced at higher concentrations. However, it must still be noted that Simpson *et al.* (1999) observed significantly decreased bacterial numbers over all elafin concentrations, as compared with bacterial growth in controls, and these findings are also in disagreement with those of Meyer-Hoffert *et al.* (2003). A critical difference between the studies, and one not taken into account in the discussion of the paper by Meyer-Hoffert *et al.*, is that the elafin peptides used by the two groups were different; while the study by Simpson *et al.* used synthetic full-length elafin (95 amino acids), the NH_2-terminal domain (50 amino acids) and the COOH-terminal domain (45 amino acids), Meyer-Hoffert *et al.* used a recombinant 57 amino acid form of elafin consisting predominantly of elafin's COOH-terminal domain (Wiedow

et al., 1990; Meyer-Hoffert *et al.*, 2003). Nevertheless, Simpson *et al.*'s study additionally demonstrated an inverse dose-response relationship for the antimicrobial activity of the COOH-terminal domain against *P aeruginosa*, adding further to the intriguing comparison between these studies. Neither study provided evidence of a microbicidal effect of elafin against *P. aeruginosa* i.e. a reduction in bacterial numbers below those added at the outset of the assays.

Therefore, it seems reasonable to suggest that the findings of our study bear a stronger relationship with those of Simpson *et al.* (1999). Importantly, the elafin protein secreted by Ad-elafin-infected cells is the same as the 9.9kDa (95 amino acid) full-length synthetic elafin peptide used in that study. As mentioned, while this previous investigation demonstrated an increase in bacterial numbers as elafin concentration increased, at no point did the number of PAO1 colonies exceed those in controls (Simpson *et al.*, 1999). Here, Ad-elafin-infection of tracheal epithelium resulted in impairment of cellular clearance of PAO1 (and indeed J1385), coupled with a significant augmentation of bacterial survival compared with controls (Figures 4-6). While it must be pointed out that Ad-lacZ infection generally caused a slight hindrance of cellular bactericidal activity, these effects were not significant (Figures 4-6).

The observation that Ad-elafin-infected cells encourage growth of *P.aeruginosa* is most interesting, and the reasons for this phenomenon are not entirely clear.

One potential explanation could be that this organism is capable of using elafin as a nutrient for growth, and is therefore able to thrive in the airway surface liquid of cells secreting elafin. *P. aeruginosa* is known to have both a broad metabolic diversity, for example its use of crude oil hydrocarbons as an energy source in the environment, and a propensity to survive and grow in the presence of limited nutritional resources (Juffs, 1976; Clarke, 1982; Terry *et al.*, 1991; Alonso *et al.*, 1999). In seeking an explanation for the inverse dose-response relationship observed during their investigations, Simpson *et al.* (1999) suggested that there may

exist a critical balance between elafin exerting an antimicrobial effect at lower concentrations, and providing a source of nutrition for *P. aeruginosa* at higher concentrations. This hypothesis may also be applicable to our studies. Elafin levels recovered from the airway surface fluid of Ad-elafin-infected cells were typically around 3.5μg/ml, equivalent to 350nM elafin (Figure 3) (this figure in itself can only be approximate, since in its calculation we assumed the total volume of ASL bathing cells to be 1μl). Although this concentration would be at the low end of the range used by Simpson *et al.* (the lowest concentration assayed in their study was 1μM), the very small volume of ASL produced by cells may mean that extracellular elafin is in fact highly concentrated on the epithelial cell surface. Considerable differences in the numbers of bacteria used in the two studies also make direct comparisons difficult, but it seems plausible that *P. aeruginosa* may be able to thrive on the high concentrations of elafin secreted by Ad-elafin-infected cells. Unfortunately, constraints dictated that we have no data pertaining to the levels of elafin recovered from Ad-elafin-infected tracheal epithelium incubated with *P. aeruginosa.* The enhanced survival of bacteria in the presence of elafin observed here does, however, add further substance to our earlier hypothesis to explain disappearance of the elafin band produced by Ad-elafin-infected Clara cells on incubation with PAO1 (Figure 1). The complete 'removal' of detectable elafin by PAO1 from the culture media of Ad-elafin-infected cells may be considered even more remarkable in view of the fact that Gram-negative LPS has previously been shown to up-regulate elafin production by Ad-elafin-infected cells (Simpson *et al.*, 2001a). Additionally, it is possible that elastase produced by *P. aeruginosa* could inactivate elafin directly, as has previously been demonstrated in the case of the other member of the ALP superfamily, SLPI (Sponer *et al.*, 1991).

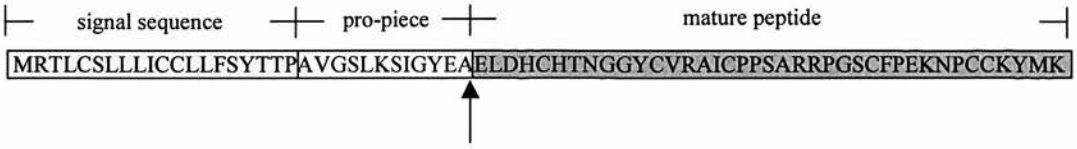
Interestingly, these findings may help to explain the previous observation that elafin levels are down-regulated in sputa from cystic fibrosis patients (Sallenave *et al.*, 1999b). The presence in sputum of large numbers of *P. aeruginosa*, and potentially other pathogens such as the pseudomonad *Burkholderia cepacia*, may lead to degradation of elafin *in vivo*, and thus contribute to enhanced colonisation and reduced antimicrobial activity against organisms such as *S. aureus*. SLPI levels

were contrastingly shown to be increased in CF sputa (Sallenave *et al.*, 1999b), and therefore it would be of interest to investigate the effects of adenoviral overexpression of SLPI on the antibacterial activity of tracheal epithelium in the same model used here.

In concert with the aforementioned hypotheses to explain increased *P. aeruginosa* survival, adenoviral gene transfer of elafin to tracheal epithelium may potentially affect the cellular production of endogenous antimicrobial peptides. The murine lung is known to produce a range of cationic antimicrobial peptides known as the murine β-defensins (Huttner *et al.*, 1997; Bals *et al.*, 1998a; Morrison *et al.*, 1998; Morrison *et al.*, 1999; Bals *et al.*, 1999a; Jia *et al.*, 2000; Bauer *et al.*, 2001; Yamaguchi *et al.*, 2001; Morrison *et al.*, 2003); in several cases expression of these peptides has been specifically demonstrated in the trachea (Bals *et al.*, 1998a; Morrison *et al.*, 1998; Morrison *et al.*, 1999; Jia *et* al., 2000; Yamaguchi *et* al., 2001). Murine β-defensins have been shown to possess antimicrobial activity against Gram-positive and Gram-negative bacteria, including *P. aeruginosa* and *S. aureus*, and therefore any disruption in the activity of these peptides could potentially weaken the antimicrobial profile of airway epithelium (Bals *et al.*, 1998a; Morrison *et al.*, 1998; Bals *et al.*, 1999a; Bauer *et al.*, 2001; Yamaguchi *et al.*, 2001; Morrison *et al.*, 2002a; Morrison *et al.*, 2002b).

Posttranslational proteolytic processing is required in order to convert β-defensins from a precursor (prepropeptide) form into their mature antimicrobial form (Martin *et al.*, 1995; Lehrer and Ganz, 2002). Other cationic antimicrobial peptides also rely on proteolytic cleavage for liberation of the fully functional molecule; for example, cryptdins of murine Paneth cells are processed by the matrix metalloproteinase matrilysin, while neutrophilic cathelicidins such as porcine protegrin-3 and human LL-37 require extracellular processing by the serine proteases elastase and proteinase-3 respectively (Panyutich *et al.*, 1997; Wilson *et al.*, 1999; Sorensen *et al.*, 2001). With regards in particular to the last two examples, it is apparent that epithelial overexpression of a serine proteinase inhibitor such as elafin may potentially inhibit cleavage of endogenous antimicrobial peptides to their

mature form. Although little is currently known about the enzymes required for processing of murine β-defensins, serine proteinases such as those inhibited by elafin could potentially be involved. As discussed in Chapter 1, elafin has a narrow spectrum of protease inhibitory activity and in humans has been shown to inhibit only neutrophil elastase (HNE) and proteinase-3 (Wiedow *et al.*, 1990; Sallenave and Ryle, 1991; Wiedow *et al.*, 1991). While these proteases are well characterised as neutrophilic enzymes, epithelial cells may also be a source of proteinase-3 or elastase-like activity (Schwarting *et al.*, 2000; Achilles and Bednarski, 2003). HNE preferentially hydrolyses bonds adjacent to small aliphatic amino acids such as valine or alanine, while proteinase-3 preferentially cleaves alanine, serine and valine (Bode *et al.*, 1989; Rao *et al.*, 1991). Analysis of peptide sequence alignments of murine β-defensins (provided by Morrison *et al.* (2003)) reveals that several peptides, including murine β-defensin-2, have an alanine residue adjacent to the bond cleaved to form the mature peptide (see murine β-defensin-2 sequence below this paragraph; putative cleavage site indicated by an arrow); such sites could potentially be a target for elastolytic activity. Therefore, it is also feasible that Ad-elafin infection of murine epithelium could hinder the innate ability of cells to kill *P. aeruginosa* by inhibiting processing of endogenous antimicrobial peptides. However, such a hypothesis is entirely speculative and warrants further investigation. It would thus be of interest in future studies to investigate levels of defensin production by these tracheal epithelia models, prior to and following infection with Ad-elafin, to determine whether antiproteinase overexpression can interfere with their processing (and function).



Finally, it has previously been demonstrated that serine proteinase inhibitors such as α1-proteinase inhibitor and α1-antichymotrypsin can interact with and inactivate the α-defensin human neutrophil peptide (HNP)-1 (Panyutich *et al.*, 1995). The same study also observed that SLPI did not interact with HNP-1, and hypothesised that this may be due to the cationic nature of both molecules. While elafin has a similar cationic character to SLPI, and a direct interaction between elafin

and defensins thus seems unlikely, any complex formation between elafin and endogenous cationic antimicrobial peptides could have potentially compromised bacterial killing in these experiments.

It is important to note that these assays, while providing an interesting *ex vivo* model for the effects of Ad-elafin on the antimicrobial activity of murine tracheal epithelium against *P. aeruginosa*, are indubitably insufficient to recreate the complex interactions which take place during bacterial infection *in vivo*. This is highlighted by comparison with the study performed by Simpson *et al.* (2001), which demonstrated that adenoviral augmentation of elafin in the murine lung was protective against acute injury mediated by *P. aeruginosa* PAO1, and resulted in significantly reduced bacterial numbers in the airways. Evidently the immune responses employed to combat and clear *P. aeruginosa* infection *in vivo* are far more complex than can be modelled in our system, and the effects of elafin augmentation in the host are more extensive than can be attributed to its antimicrobial activity alone.

6.3.2. The effects of elafin gene augmentation on the antimicrobial activity of primary murine tracheal epithelium against *S. aureus*

In contrast to the results obtained with the Gram-negative organism *P. aeruginosa*, Ad-elafin infection significantly enhanced the antimicrobial activity of murine tracheal epithelium against the Gram-positive organism *S. aureus* C1705 (Figures 7-9). Also of interest was the finding that tracheal epithelium was unable to exert a bactericidal effect against C1705, such that bacterial numbers typically increased by an average of around 20-fold following 3-hour incubation with cellular inserts (Figure 7). This suggests that production of endogenous defensins by tracheal epithelium, such as murine β-defensins 1, 2, 4 and 6 (Bals *et al.*, 1998a; Morrison *et al.*, 1998; Morrison *et al.*, 1999; Jia *et al.*, 2000; Yamaguchi *et al.*, 2001), may be insufficient to kill *S. aureus* C1705, despite demonstrable activity of at least murine β-defensin 1 against *S. aureus in vitro* (Bals *et al.*, 1998a). The mechanism of *S. aureus* resistance to defensin activity in tracheal cultures may be complex, but could be due to its capacity to modify phosphatidylglycerol, one of its major membrane phospholipids, with *L*-lysine, giving the molecule a net positive charge and thus

resistance to cationic defensins (Peschel, 2002). A recent study has also described an exoprotein produced by *S. aureus*, known as staphylokinase, which can bind to and neutralise the antimicrobial activity of human α-defensins (Jin *et al.*, 2004). This strain of *S. aureus* can, however, be cleared effectively by murine lungs *in vivo* (Morrison *et al.*, 2002a).

The effects of elafin in this model cannot be described as bactericidal, since the cfu of *S. aureus* recovered following incubation with Ad-elafin-infected cells were not lower than those added at the outset (Figure 7). However, compared to uninfected and Ad-lacZ-infected cells, cells producing elafin exerted a considerably enhanced antimicrobial activity (Figures 8 and 9). Elafin secretion is therefore associated with significant inhibition of growth of *S. aureus*, although it is unclear from these studies whether elafin acts to slow the growth of the organisms, directly kills a proportion of the organisms, or a combination of both. Moreover, a synergistic activity of elafin with endogenous antibacterial peptides (which may be inadequate to combat infection alone) cannot be ruled out; synergism between neutrophil defensins and cathelicidins has previously been demonstrated against *S. aureus* (Nagaoka *et al.*, 2000).

Cationic antimicrobial peptides can interact directly with components of *S. aureus*, such as teichoic acid and lipoteichoic acid (Scott *et al.*, 1999a; Vorland *et al.*, 1999), and it is tempting to speculate that elafin may also be capable of direct interaction with similar structural determinants of Gram-positive bacteria. Elafin can be included among a wide range of antimicrobial peptides which have been shown to display antimicrobial activity against *S. aureus* (Giacometti *et al.*, 1998; Severina *et al.*, 1998; Turner *et al.*, 1998; Friedrich *et al.*, 2000; Travis *et* al., 2000; Midorikawa *et al.*, 2003). SLPI, the other member of the antileukoprotease superfamily of proteinase inhibitors, has also been demonstrated to possess antimicrobial activity against several common bacterial pathogens, including *S. aureus* and *P. aeruginosa* (Hiemstra *et al.*, 1996; Wiedow *et al.*, 1998).

With regards to the adenoviral gene transfer techniques used to confer elafin production to epithelial cells in our investigations, recent studies have demonstrated similar enhancement of antimicrobial activity by overexpression of the human cathelicidin antimicrobial peptide hCAP18/LL-37. While administration of a synthetic hCAP18/LL-37 peptide could protect mice against the effects of *P. aeruginosa* (Kirikae *et al.*, 1998), overexpression using adenovirus vectors containing the cDNA for hCAP18/LL-37 were shown to significantly augment bacterial killing in two separate studies (Bals *et al.*, 1999b; Bals *et al.*, 1999c). *In vitro*, adenoviral overexpression of this cathelicidin in a cystic fibrosis bronchial xenograft model augmented the antimicrobial activity of ASL against both *S. aureus* and *P. aeruginosa* (Bals *et al.*, 1999b). In mouse models, intratracheal administration of these adenoviral vectors conferred protection against the effects of pulmonary challenge with *P. aeruginosa*, and was associated with a lower bacterial load (Bals *et al.*, 1999c). It is therefore apparent that adenoviral gene transfer of antimicrobial peptides may provide protection against certain bacterial pathogens.

A further interesting observation made during our studies was that incubation of *S. aureus* with Ad-elafin-infected tracheal epithelium actually enhanced secretion of elafin (Figure 10). This also contrasts with the previous finding that *P. aeruginosa* appeared to eradicate elafin secreted by cells (Figure 1), perhaps reflecting elafin-mediated antimicrobial action against *S. aureus* on one hand and metabolism of elafin by *P. aeruginosa* on the other. Although the mechanisms by which *S. aureus* upregulates elafin secretion are unclear, it may be possible that components of this organism, such as lipoteichoic acid, can regulate elafin transgene expression in a similar fashion to that demonstrated by LPS (Simpson *et al.*, 2001a). As per Simpson *et al.* (2001a), up-regulation may be due to stimulatory effects of bacterial products on the mCMV promoter at the transcriptional level; however, no mRNA data was obtained in order to support this hypothesis.

6.4. SUMMARY

The work in this chapter has demonstrated that Ad-elafin infection of primary murine tracheal epithelium can modulate the antimicrobial activity of these cells against common respiratory pathogens. However, the effects of Ad-elafin were highly dependent upon the type of organism used to model infection. Ad-elafin infection conferred significant antimicrobial activity against the Gram-positive organism *S. aureus*, but compromised the inherent ability of cells to kill Gram-negative *P. aeruginosa*, and in fact greatly enhanced its survival.

While Bals *et al.* (1999b) demonstrated that adenoviral overexpression of the cathelicidin hCAP18/LL-37 could reverse the bacterial killing defect against both *S. aureus* and *P. aeruginosa* in a cystic fibrosis bronchial xenograft model, the data presented here suggest that the therapeutic advantage of elafin augmentation may be more pathogen-specific. Elafin overexpression may be of benefit in eliminating pathogens in conditions such as CF when the spectrum of pathogens is clearly defined, for example in infancy prior to colonisation with *P. aeruginosa*; however, caution is undoubtedly warranted in the design of treatments for such a complex clinical condition.

In conclusion, these findings contribute to the knowledge surrounding potential roles for elafin in the innate immune response to bacterial infection, and emphasise the need for further characterisation of the effects of elafin overexpression *in vivo*.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The experiments described in this thesis have shown that elafin can bind directly to Gram-negative bacterial lipopolysaccharide, a potent immunomodulatory molecule and key instigator of the pathophysiology of Gram-negative septic shock. Furthermore, binding of elafin to LPS appears to occur within the conserved lipid A-core portion of the LPS molecule, suggesting that elafin may recognise a broad range of bacterial lipopolysaccharides. Elafin bound to LPS can prevent subsequent interaction of LPS with the serum lipid transfer protein LBP, a critical step in LPS-mediated cellular activation.

This observation led to experiments suggesting that binding of elafin to LPS may provoke contrasting roles in inflammatory immune responses. In the presence of serum, exogenous elafin dampened LPS activation of macrophages, whereas LPS-induced responses were augmented by elafin in the absence of serum. These findings have suggested that inhibition of LPS interaction with serum factors such as LBP may confer to elafin a net anti-inflammatory role in macrophage activation while, in conditions short of serum reactants, elafin may itself function as an LPS 'transfer' molecule.

Elafin gene augmentation was found to suppress the inflammatory response of macrophages to LPS in serum-containing milieu, and further experiments demonstrated that these effects were not mediated by extracellular elafin. These findings infer an important role for intracellular elafin in dampening macrophage responsiveness by interfering with the LPS signalling pathway.

Together these observations suggest that elafin may fulfil a dual role in innate immunity, enhancing immune responses in sites of local inflammation, such as the airways, but dampening potentially deleterious systemic inflammatory responses in the circulation.

The characteristics of elafin suggest intrinsic antimicrobial properties, and adenovirus-mediated elafin overexpression in murine tracheal epithelium *ex vivo* was shown to confer antimicrobial activity against *Staphylococcus aureus*, but compromised the inherent ability of cells to kill *Pseudomonas aeruginosa*. Ad-elafin infection may thus confer pathogen-specific augmentation of antimicrobial defences in cells constantly exposed to microbial challenge.

These experiments advance our understanding of elafin biology and of potential activities for elafin in modulating inflammation *in vivo*, but a great many findings require further definition and could form the subject of future research.

It would be of interest to determine whether elafin can also inhibit interactions of LPS with other molecules of the LPS receptor complex, such as CD14, or indeed prevent formation of the LPS-CD14-TLR4-MD-2 complex. Such inhibitory activities could potentially contribute to the anti-inflammatory effects of elafin on macrophage activation in the presence of serum. However, since elafin was found to play divergent roles in LPS-mediated activation, a potential role for elafin in accelerating binding of LPS to CD14 or to TLR4-MD-2 is also a possibility, and should be addressed. As previously suggested in Chapter 4, the use of adenovirus techniques to overexpress TLR4-MD-2 and CD14, perhaps even in cells which do not normally express these molecules, may be of benefit in investigating elafin's putative function in facilitating LPS transfer to the receptor machinery. Additionally, experiments aimed at investigating the key serum factors that contribute to elafin acting either as LPS agonist or antagonist may involve blockade of LBP and/or sCD14 in serum, but could equally encompass cellular overexpression of these molecules in serum-free conditions.

Although not performed here, an investigation of the kinetics of elafin-LPS interaction would be useful in order to compare specifically the LPS-binding affinities of elafin with those of the LPS receptor complex. The identification of a putative cellular elafin receptor would also contribute to knowledge surrounding roles for elafin during inflammation, since elafin uptake could potentially dampen

LPS-mediated inflammation intracellularly, or lead to elafin-LPS internalisation. Furthermore, identification of such an elafin receptor may also provide a mechanism by which elafin can mediate its chemotactic activity for neutrophils; for example, the human cathelicidin LL-37 has been shown to use the formyl peptide receptor-like 1 (FPRL1) to chemoattract neutrophils, monocytes and T cells (Yang *et al.*, 2000).

In terms of the effects observed in this study of elafin gene augmentation in macrophages, creation and overexpression of a non-secretory form of elafin could potentially help to answer questions regarding its intracellular role in modulating LPS responses. Likewise, it would be of interest to mutate or oxidise elafin's active site in order to investigate whether antiproteinase activity is important for the anti-inflammatory effects observed here. Elafin's effects on components of the LPS signalling pathway upstream of IκB, such as MyD88, IRAK and the IKK complex, also remain to be elucidated.

With regard to elafin's antimicrobial activity, it would be of interest to investigate potential synergism with other cationic antimicrobial peptides, and even with conventional antibiotics. Elafin could potentially bind systemic LPS released by bacteria following antibiotic-mediated killing and prevent subsequent inflammation, as shown previously using co-administration of BPI with antibiotic treatment. Interactions of elafin with cell surface components of Gram-positive bacteria, such as lipoteichoic acid, also merit investigation. Moreover, the effects of adenovirus-mediated cellular overexpression of elafin on the production of endogenous antimicrobial peptides, as discussed in Chapter 6, is worthy of further attention to help explain the findings made here. The potential salt-sensitivity of elafin's antimicrobial action additionally remains to be clarified, since this could abolish the molecule's activity *in vivo* in inflammatory conditions such as cystic fibrosis.

In conclusion, the findings described in this thesis may further contribute to the understanding of roles played by elafin in the innate immune response. The activities ascribed to elafin thus far, including antiproteinase, antimicrobial, neutrophil-chemoattractant and anti-endotoxin activities, have suggested a range of

functions for this molecule in mediating inflammatory responses. Due to its widespread distribution within skin, mucosal epithelial tissues and cells of myeloid lineage, and upregulation in response to early inflammatory stimuli, elafin may be ideally placed to fulfil a sentinel role as a multi-functional modulator of innate immune responses.

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