Bacterial Lipopolysaccharide and Tumour Necrosis Factor- alpha Synergism in Inflammation.

Hubertus Paul Anton Jersmann

M.B.B.S.Düsseldorf, M.D., F.R.A.C.P.

Thesis submitted for the degree of Doctor of Philosophy

Department of Immunopathology

Women's and Children's Hospital

&

Department of Paediatrics

University of Adelaide

Faculty of Medicine

December 2000

Abstract

During infection with bacteria exogenous and endogenous mediators combine to form a complex network. Although the biological activities of individual pro-inflammatory agonists are well characterized, there is an incomplete understanding of the interactions between different mediators.

In order to expand the knowledge of mediator-interactions this thesis examines different aspects of 'cross-talk' between bacterial lipopolysaccharide (LPS) and tumour necrosis factor-alpha (TNF-α) *in vitro* in relation to the respiratory epithelium, vascular endothelium, monocytes/macrophages and neutrophils.

Monocytes and macrophages responded to co-stimulation with LPS and TNF-α with an increased production of proinflammatory cytokines. Neutrophils were primed by pretreatment with TNF-α for an enhanced LPS-induced respiratory burst, and also showed a synergistic increase in their adhesive properties. Human umbilical vein vascular endothelial cells (HUVEC) responded with the synergistic upregulation of the adhesion molecules E-selectin, ICAM-1 and VCAM-1 when treated with TNF-α and LPS. In a human alveolar type II respiratory epithelial cell line (A549) TNF-α upregulated the expression of the adhesion molecule ICAM-1, whereas LPS had no effect. However, in concert with IFN-γ or a cocktail of cytokines (IL-1β, TNF-α and IFN-γ) LPS had an enhancing effect on ICAM-1 upregulation.

The mechanisms of the synergistic effects of LPS and TNF-α were investigated. The LPS receptor CD14 on the surface of neutrophils was upregulated by TNF-α, which correlated with an increase in LPS-binding, possibly at least in part accounting for the priming effect by TNF-α. Interstingly, while it is believed that endothelial cells are CD14-negative our studies showed that these cell express CD14. Expression of CD14 on HUVEC could be modulated and was dependent on protein synthesis. The incorporation of radioactive amino acid into CD14 confirmed it to be of endothelial origin. Further work was carried out to determine why CD14 had not been detected previously. Functional studies revealed that cell-associated CD14 is required for LPS-induced endothelial cell activation, while serum factors act as enhancers. CD14 was detected on the A549 and 16HBE14o- respiratory epithelial cell lines, which also had not been previously described. However, because these cells are relatively insensitive to LPS, and the binding of LPS to these cells is CD14-independent, the relevance of this finding is not yet clear.

To understand how the synergism between LPS and TNF-α may operate, the intracellular signalling pathways stimulated by these mediators were examined in detail in HUVEC. Synergism between the two pathways was found to be due to increased transcription. Enhanced activation of the transcription factor NF-κB and, to a lesser extent, the MAP-kinases p38 and JNK were demonstrated.

This thesis has contributed to the knowledge of how the bacteriokine-cytokine network operates by demonstrating how two major proinflammatory mediators interact in modulating the inflammatory response. Furthermore the discovery of CD14 expression on endothelial cells not only provides greater insight in the pathogenesis of bacterial infection, sepsis and perhaps atherosclerosis, it is also likely to influence the future development of new treatment strategies for those conditions.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

.... 30. 12. 2000

Hubertus P. A. Jersmann   Dated

Acknowledgements

Publications, Presentations, Scholarships and Awards

The following work was carried out as part of my thesis and has been published or been accepted for publication:

Jersmann, H.P.A., Rathjen, D.A. and Ferrante, A. 1998. Enhancement of Lipopolysaccharide-induced neutrophil oxygen radical production by tumor necrosis factor alpha. *Infect. Immun.* **66**:1744-1747.

Jersmann, H.P.A., Hii. C.S.T., Hodge, G.L. and Ferrante, A. 2001. Synthesis and surface expression of CD14 by human endothelial cells. (*Infect. Immun.*, publishing date January 2001)

Jersmann, H.P.A., Hii, C.S.T., Ferrante, J.V. and Ferrante, A. 2001. Bacterial lipopolysaccharide and tumour necrosis factor-alpha synergistically increase expression of human endothelial adhesion molecules through activation of NF-κB and p38 MAP kinase signaling pathways. (*Infect. Immun.*, publishing date March 2001)

The following work was also conducted as part of the thesis and is currently prepared for publication:

Jersmann, H.P.A. and Ferrante, A. 2001. Synergistic responses of mononuclear phagocytes to tumor necrosis factor and bacterial lipopolysaccharide.

Jersmann, H.P.A. and Ferrante, A. 2001. Effects of tumor necrosis factor and LPS on ICAM-1 expression in Respiratory epithelial cells.

Other work which is being prepared for publication but will not be reported in this thesis:

Robinson, B.S., Ferrante, A., Jersmann, H.P.A., Hii, C.S.T., Parashakis, G., Ferrante, J.V., Huang, Z.H., Trout, N.A., Pitt, M.J., Rathjen, D.A., Easton, C.J., Poulos, A., Murray, A.W. and Prager, R.H. 2000. A potential new class of pharmaceuticals and research tools: engineered fatty acids which regulate transcription factors and inhibit expression of endothelial adhesion molecules and inflammation. (submitted *J.Immunol.*, December 2000)

Moghaddami, N., Jersmann, H.P.A., Huang, Z.H., Hii, C.S.T. and Ferrante, A. 2001. The Omega-3 polyunsaturated fatty acid AA induces upregulation of TNF receptor expression in human neutrophils. (In preparation)

Some of the work described in this thesis was presented at the following scientific meetings either in poster or abstract format:

Australian Society for Medical Research (ASMR), 1997

Australian Society for Imunology (ASI), Annual Scientific Meeting, Adelaide, 1998

Australian Society for Vascular Biology (AVBS), Annual Scientific Meeting, Adelaide, 1999

Young Investigator Award Presentations, South Australian Branch of the Thoracic Society of Australia and New Zealand (TSANZ), Adelaide, 1999

British Society of Immunology, Scottish Branch ASM, St Andrews, UK, 2000

Scholarships and awards:

Reginald Walker scholarship for medical graduates   (1995)

Sanofi-Winthrop Travel scholarship   (1999)

TSANZ SA Young Investigator Award   (1999)

Table of Contents

Abbreviations

Å	Ångström, 10^{-10}m	CD11c/CD18	CR4, ICAM-1 ligand, binds fibrinogen
AA	Arachidonic acid		
AAD	Aminoactinomycin-D	CD14	LPS receptor
ABTS	Buffer, 2, 2'-azino-di-[3 ethyl-benzthiozolin-sulfonate(6)]	CD15s	Sialyl Lewis X, E-selectin ligand
		CD29/CD49d	VLA-4
		CD33	Monocyte surface marker
ADP	Adenosine-di-phosphate	CD51	Vitronectin receptor
ANOVA	Analysis of variance	CD54	ICAM-1
AP-1	Activator protein-1	CD58	LFA-3
APS	Ammonium persulfate	CD62L	L-selectin
ARDS	Adult respiratory distress syndrome	CD62P	P-selectin, GMP140
		CD63	gp53
ASK-1	Apoptosis-signalling kinase-1	CD106	VCAM-1, VLA-4, $\alpha_4\beta_7$ integrin-ligand
ATCC	American tissue culture collection		
		cDNA	Complementary DNA
ATF-2	Activator transcription factor-2	C1, C2, C3, C4	Complement components
		Ci	Curie
ATP	Adenosine triphosphate	CHO	Chinese hamster ovary (tumour cell line)
BALT	Bronchus-associated lymphoid tissue		
		CHX	Cycloheximide
BPD	Broncho-pulmonary dysplasia	CL	Chemiluminescence
		CPM	Counts per minute
BPI	Bactericidal/permeability-increasing protein	CR3	CD11b/CD18
		CSF	Colony stimulating factor
BSA	Bovine serum albumin	DAB	3,3'-di-amino-benzidine
CAPK	Ceramide-activated protein kinase	DIC	Disseminated intravascular coagulation
CD	Cluster designation number	DMSO	Dimethylsulfoxide
CD2	LFA-2	DT	Diphtheria toxin
CD11a/CD18	LFA-1, ICAM-1 ligand	DTT	DL-Dithiothreitol, Cleland's reagent
CD11b/CD18	MAC-1, CR3, ICAM-1 ligand		
		EC	Endothelial cells
		ECGF	Endothelial growth supplement

EDTA	Ethylenediaminetetraacetic acid	HSA	Human serum albumin
ELISA	Enzyme-linked immunosorbent assay	HSP	Heat shock protein
		hTLR2,4	Human TOLL-like receptors 2, 4
EGF	Endothelial growth factor	HUS	Haemolytic Uraemic Syndrome
ERK	Extracellular-signal regulated kinase		
		HUVEC	Human umbilical vein Endothelial cells
E-selectin	Endothelial leukocyte adhesion molecule-1 (ELAM-1), CD62E	ICAM-1	Intercellular adhesion molecule-1,CD54
FACS	Fluorescence activated cell sorter		
FAD	A flavocytochrome, part of the NADPH complex	ICE	Interleukin-1 converting enzyme
FADD	Fas-associated death domain	IFN-γ	Interferon gamma
FCS	Fetal calf serum	IgA	Immune globulin A
FITC	Fluorescein isothiocyanate	IgG	Immune globulin gamma
fMLP	N-formyl-L-methionyl-L-leucyl-L-phenylalanine	IκB	Inhibitor of NF-κB
		IKK	IκB kinase
GAPDH	Guanosin-adenosin-phosphate dehydrogenase	IL-1	Interleukin-1
GAS	γ-activation site	IL-1 AcP	Interleukin-1 receptor-associated protein
GCK	Germinal center kinase		
GCKR	Germinal center kinase-related	IL-2	Interleukin-2
		IL-3	Interleukin-3
GM-CSF	Granulocyte/macrophage colony stimulating factor	IL-4	Interleukin-4
		IL-6	Interleukin-6
GPI	Glycosylphosphatidylinositol	IL-8	Interleukin-8
GTP	Guanosine triphosphate	IL-10	Interleukin-10
HBEC	Human bronchial epithelial cells	IL-11	Interleukin-11
		IL-12	Interleukin-12
HBSS	Hanks' balanced salt solution	iNOS	Inducible nitric oxide synthetase
HETE	Hydroxyeicosatetranoate	IRAK	Interleukin-1 receptor-associated kinase
HIV	Human immune deficiency virus		
		JAK	Janus kinase
HLA	Human lymphocyte antigen	JNK	c-jun amino terminal kinase
HOCl	Hypochlorous acid	KCl	Potassium chloride
HRP	Horse radish peroxidase	kD	Kilodalton

LPA	Lisophosphatidic acid		
LAM	Lipoarabinomannan		inducing kinase
LAP	Lipid A-associated protein	NK	Natural killer cell
LBP	Lipoprotein binding protein	NO	Nitric oxide
LFA-1	Lymphocyte function associated	NOS	Nitric oxide synthetase
	antigen-1, CD11a/CD18	OD	Optical density
LM	Lipomannan	ODRS	Oxygen-derived reactive species
LPS	Lipopolysaccharide	PAF	Platelet activating factor
LTA	Lipoteichoic acid	PAGE	Polyacrylamide gel
LTB$_4$	Leukotriene B$_4$		electrophoresis
mab	Monoclonal antibody	PAI	Plasminogen activator inhibitor
MAC	Membrane attack complex	PBS	Phosphate buffered solution
Mac-1	CD11b/CD18, CR3	PDGF	Platelet-derived growth factor
MAP	Mitogen activated protein	PE	Phycoerythrin
MAP-kinase	Mitogen activated protein kinase	PIM	Phosphatidylinositol mannosides
MC	Mononuclear cells	PIPLC	Phosphatidylinositol-specific
MCP-1	Monocyte chemotactic protein-1		phospholipase C
MEK	MAP ERK kinase	PIPLD	Phosphatidylinositol-specific
MEKK	MAP ERK kinase kinase		phospholipase D
MFI	Mean fluorescence intensity	PKC	Protein kinase C
MHC	Major histocompatibility complex	PLA$_2$	Phospholipase A$_2$
MKK	MAP kinase kinase	PLD	Phospholipase D
MKKK	MAP kinase kinase kinase	PMA	Phorbol 12-myristate 13-acetate
MnSOD	Manganese superoxide dismutase	PMSF	Phenylmethylsulfonyl fluoride
Moesin	Membrane-organizing	PNH	Paroxysmal nocturnal
	extension-spike protein		haemoglobinuria
mRNA	Messenger ribonucleic acid	Pyk-2	Proline-rich tyrosine kinase-2
MW	Molecular weight	RANTES	Regulated upon activation, normal
MyD88	Myeloid differentiation		T-cell expressed, presumed secreted
	antigen 88	RIP	Receptor-interacting protein
NADPH	Nicotinamide adenosine	RPMI 1640	Roswell Park Memorial Institute
	di-phosphate		(buffered culture media)
NANC	Non-adrenergic/non-	RSV	Respiratory syncytial virus
	cholinergic system	SAM	Surface-associated material
NF-κB	Nuclear factor-kappa B	SAPK	Stress activated protein kinase
NIK	Nuclear factor-kappa B-	SDS	Sodium dodecyl sulphate

SEM	Standard error of the mean	TNF-α	Tumour necrosis factor alpha
Serpin	Serine proteinase inhibitor (eg. α₁-antitrypsin)	TGF-β	Tumour growth factor-beta
		TPA	Tetradecanoyl phorbol acetate
SOD	Superoxide dismutase	TRADD	Tumour necrosis factor receptor-
SPE-B	Streptococcal pyrogenic exotoxin B		associated death domain
STAT	Signal transducer and activator of transcription	TRAF	Tumour necrosis factor receptor-associated factor
TAK	TGF-β activated kinase	UTI	Urinary tract infection
TAP	Tracheal anti-microbial peptide	UV	Ultra violet
TEMED	N,N,N'N'-tetramethylethylenediamine	VCAM-1	Vascular cell adhesion molecule-1,
TES	N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid		CD106
		VLA	Very late activation (integrin
TF	Tissue factor		family-type receptors)

Index of Figures **Page No**

Index of Diagrams   **Page No**

Index of Tables   **Page No**

Index of Photographs   Page No

Chapter 1

Introduction

1.1   GENERAL INTRODUCTION

Encounters of host tissues with microorganisms such as bacteria result in a wide range of host responses aimed at elimination of the microorganism. These protective host responses are generally referred to as 'inflammation'. The classical manifestations of inflammation have been recognized for at least 2000 years. In his famous scripts *De medicina* the Roman doctor Aulus Cornelius Celcus (30B.C.-38A.D.) described the four cardinal signs and symptoms of *rubor* (redness), *tumor* (swelling), *calor* (warmth) and *dolor* (pain). The correlates of these manifestations are dilatation of arterioles, capillaries and venules, increased blood flow, increased vascular permeability with extravasation of fluid and plasma proteins, enhanced migration of leukocytes to the site of the inflammation and the irritation of type C nociceptive nerve fibres.

Although inflammation can occur in response to foreign bodies and malignant tumours, the majority of inflammatory responses are due to invasion by microorganisms such as viruses, fungi, protozoa and bacteria. The latter and especially immuno-active mediators originating from bacteria are a main focus of this thesis.

The innate immune system serves as an immediate protection against invading bacteria and is the phylogenetically oldest part of the immune response of higher organisms (Fearon et al., 1996). Its role is to rapidly recognize and inactivate infectious agents (0-4h), and promote the development of the adaptive immune response (Diagram 1-1). Its importance is evident by the fact it has been conserved over the evolutionary period. For example, the IL-1 receptor and its numerous receptor-associated signalling molecules show significant homology with molecules in *Drosophila melanogaster* (Gay et al., 1991; Chaudhary et al., 1998; O'Neill et al., 1998; Kopp et al., 1999). The serum factors which bind LPS, LBP and CD14, are also highly conserved across different species (Grünwald, 1993).

A

B
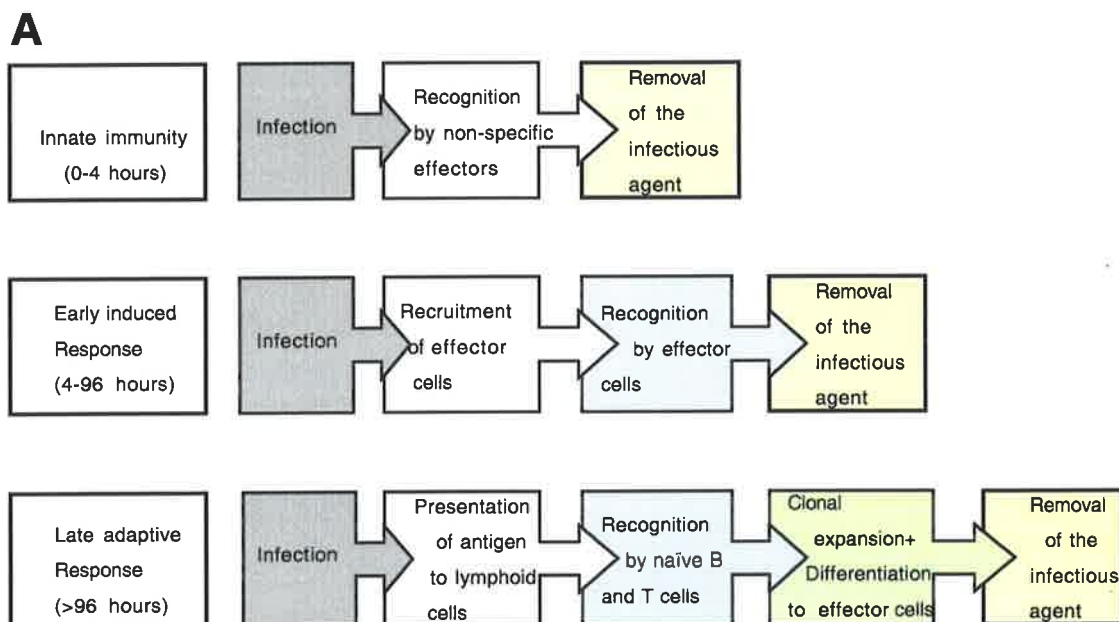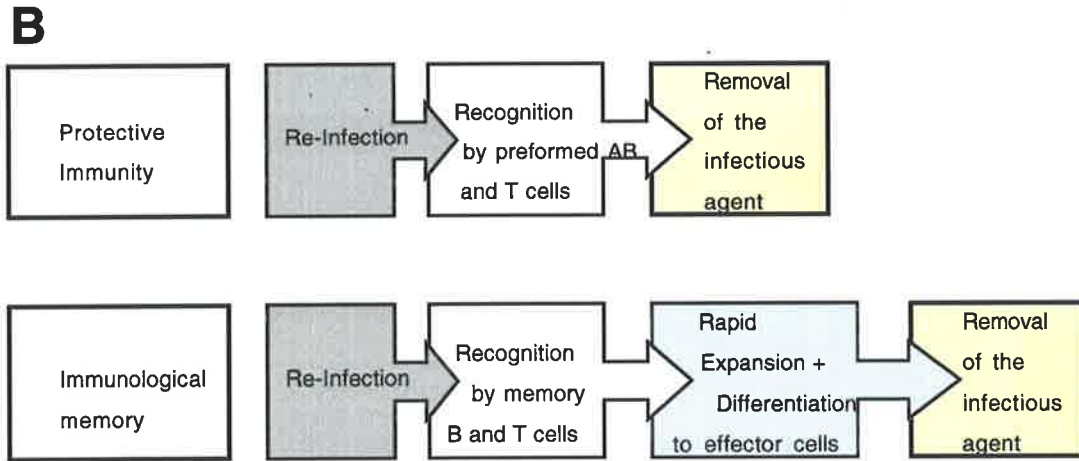
Diagram 1-1:   Synopsis of the immune responses to infectious agents. **A.** Innate immune response. There are three initial phases of acute infection during which the innate immune system facilitates immediate recognition of microorganisms, but also induces recruitment of effector cells. These facilitate late adaptive responses through macrophages presenting antigen to T cells. **B.** Protective immunity, including immunolocigal memory.

Bacteria usually invade their host via the mucosal surfaces of the respiratory, urogenital or gastrointestinal tracts, although hair follicles and breeches in the skin can also facilitate the entry of bacteria into the body. The classifications of different types of bacteria are at times confusing because they are largely derived historically. The nomenclature is based on such diverse criteria as histological staining patterns, culture conditions, virulence, surface antigens, size and shapes of light-microscopical appearance, or the diseases caused by these pathogens. Throughout this thesis bacteria shall be classified by their behaviour with gram staining, a technique first described by Christian Gram in 1884. Bacteria are stained with crystal violet, iodine solution, alcohol and safranin. Gram positive organisms retain the violet colour and appear blue, whereas gram negative organisms lose the crystal violet and counter-stain pink with safranin. This is most likely due to the fact that gram negative bacteria have a thinner cell wall with a higher lipid content than gram-positive organisms. The differences in cell wall structure between gram-positive and gram-negative bacteria are illustrated in Diagram 1-2. A list of the major gram-negative bacteria and the typical illnesses they cause is provided in Table 1-1.

A

B
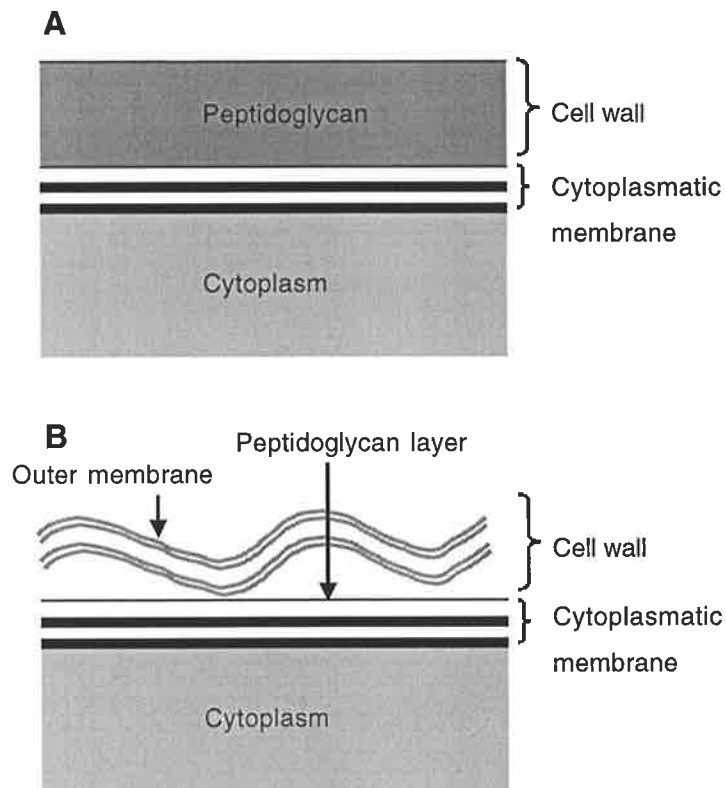
Diagram 1-2: Schematic interpretation of the structure of the cell walls of **A.** gram-positive and **B.** gram-negative bacteria, based on electron microscopic observations.

Table 1-1: Gram-negative bacteria and the illnesses they typically cause.

Gram-negative Bacteria	Disease
Pseudomonas aeruginosa	Pneumonia, bronchitis
Haemophilus influenzae	Bronchitis, Pneumonia, Otitis media, meningitis
Bordetella pertussis	Whooping cough
Klebsiella pneumoniae	Pneumonia, lung abscesses
Moraxella catarrhalis	Bronchitis
Legionella pneumophilia	Legionaire's disease, pneumonia
Mycoplasma pneumoniae	Pneumonia
Clamydia trachomatis	Trachoma
Clamydia psittaci	Psittacosis
Escherichia coli	Urinary tract infection, HUS
Yersinia pestis	Plague, pneumonia
Proteus mirabilis	Urinary tract infection
Neisseria meningitides	Meningitis, Waterhouse-Friedrichsen Syndrome
Neisseria gonorrhoeae	Gonorrhoea
Vibrio cholerae	Cholera
Enterobacter	Dysentry
Serratia marcescence	Urinary tract infection

Rickettsia prowazeckii	Rocky Mountain Spotted Fever
Salmonella typhi	Typhoid
Salmonella typhimurium	Dysentry
Shigella	Dysentry
Campylobacter jejuni	Dysentry

Following host invasion bacteria either penetrate tissues locally or disseminate to distant tissues via the blood stream (bacteriaemia). Bacteria initiate inflammation by releasing exotoxins which can activate or kill host cells. In addition damage to bacteria generates components such as endotoxins, most of which are powerful inducers of inflammation. Contact with endotoxins, exotoxins or whole bacteria leads to a multitude of responses in eukariotic cells (Diagram 1-3).
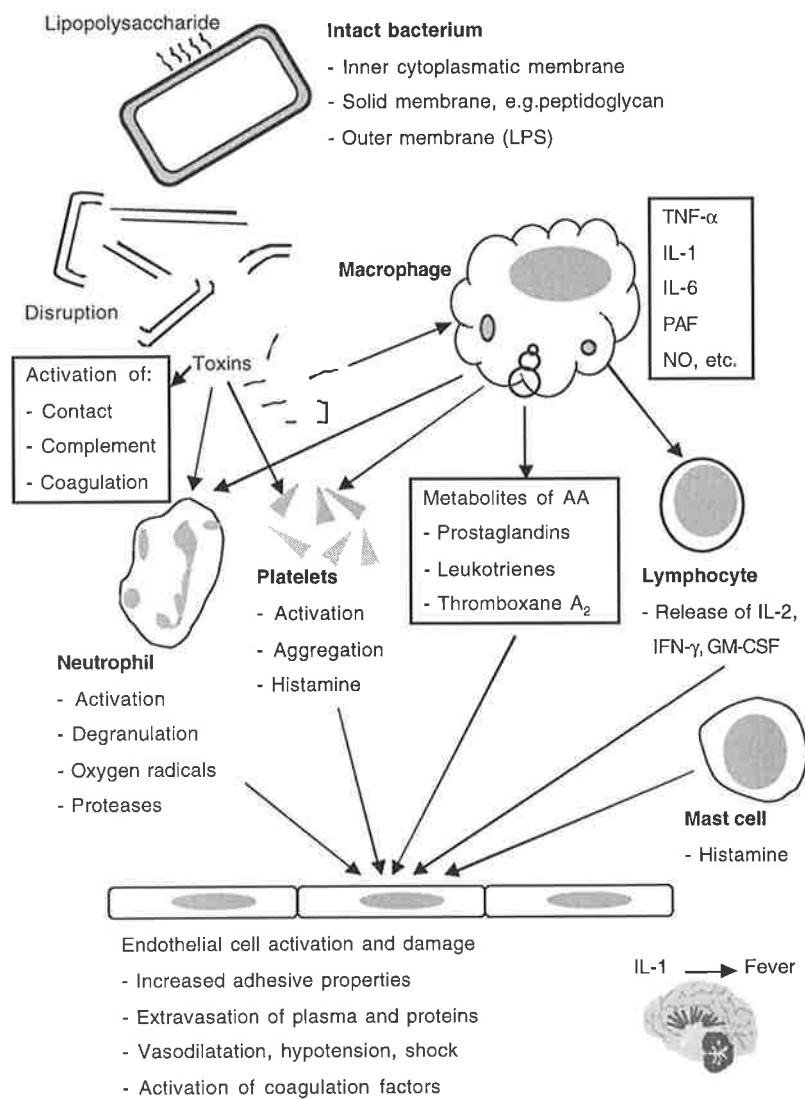
Diagram 1-3:   Schematic simplified synopsis of the effects of bacteria and their toxins on the host immune system.

Activated leukocytes release a wide variety of substances, which are directly harmful to bacteria, such as oxygen-derived reactive species and proteolytic enzymes. Additionally, leukocytes produce cytokines (Greek: κυτος, cell; κινειν, to move), small messenger molecules which recruit leukocytes to the inflammatory site and which stimulate resident cells to produce inflammatory mediators in a concerted effort to destroy bacteria.

Other groups of mediators include vaso-active substances, comprised of metabolites of the cell membrane lipid, arachidonic acid (AA), such as thromboxane A_2, prostaglandins and leukotrienes. Histamine, which is secreted by platelets and mast cells, is also a very powerful vasodilator. The complement system comprises a group of inactive proteins, which upon activation by toxins in turn activate and amplify each other in a cascade-like manner. This leads to the formation of large molecules called membrane attack complexes (MAC), which bind to bacteria and destroy them by causing breeches in the integrity of their cell wall. Another protein system is contact, which induces margination and activation of leukocytes, and the coagulation cascade.

All mediators described above, exogenous (derived from the microorganism) as well as endogenous (derived from host cells), form a complex network. There is 'cross-talk' between mediators, for example cytokines interact with complement, leukotrienes, and platelet activating factor (PAF). Also, one inflammatory agent can stimulate the production of other mediators, leading to additive effects, and, sometimes to responses much stronger than the sum of the individual stimuli, a phenomenon known as 'synergism'. Another interaction between proinflammatory substances is described as 'priming'. During priming the initial mediator does not elicit an obvious cellular response. However, it alters the target cell in such a fashion that this cell displays a much greater than anticipated response to a second stimulus. In contrast, some mediators can inhibit inflammatory responses, for example the T-cell derived cytokines IL-4, IL-10 and TGF-β downregulate the activity of macrophages (Goerdt et al., 1999).

Inflammatory agents which recruit leukocytes usually also activate barrier cells such as endothelium and epithelia to enable adhesion of those inflammatory cells and facilitate their transmigration into tissues (Huber et al., 1991). Cytokines, such as IL-1, IL-6 and TNF-α, are known to stimulate the liver to increase its production of acute phase proteins.

Occasionally microbes resist elimination, either because the organisms have adapted to the defenses of the innate immune system and withstand killing by persisting inside macrophages (e.g. in tuberculosis), or because the host has a deficiency in one of the components of the innate immune response (e.g. inability to synthesize oxygen radicals in chronic granulomatous disease). This often leads to chronic infection with chronic inflammation and characteristically macrophages accumulate in the centre of such focus, some will become confluent to form giant cells. Such formations, where macrophages are typically surrounded by activated lymphocytes, are called 'granuloma'.

1.2   CELLS INVOLVED IN THE INFLAMMATORY RESPONSE

1.2.1   Inflammatory leukocytes

There are many different types of inflammatory responses. The characteristic differences between these types is usually related to the cell types recruited at inflammatory foci. The type of microorganism invading tissue may dictate the characteristics of the inflammatory response. For example in acute inflammation and pyogenic infections neutrophils predominate. In contrast in chronic inflammation one usually encounters mononuclear infiltrates, such as T lymphocytes and macrophages which respond to intracellular parasites such as Listeria and mycobacteria. There are also inflammatory reactions in which eosinophils or basophils are the characteristic cell type, for example in helminthic infestation. The two cell types of interest in this thesis were monocytes/macrophages and neutrophils.

1.2.1.1 Monocytes and macrophages

Monocytes originate from bone marrow stem cells. They differentiate via myeloid precursor cells into pro-monocytes and migrate into the blood as monocytes. Blood monocytes settle in tissues to differentiate into macrophages, which constitute a major part of the innate immune system. The functions of monocytes and macrophages are summarized in Diagram 1-4.
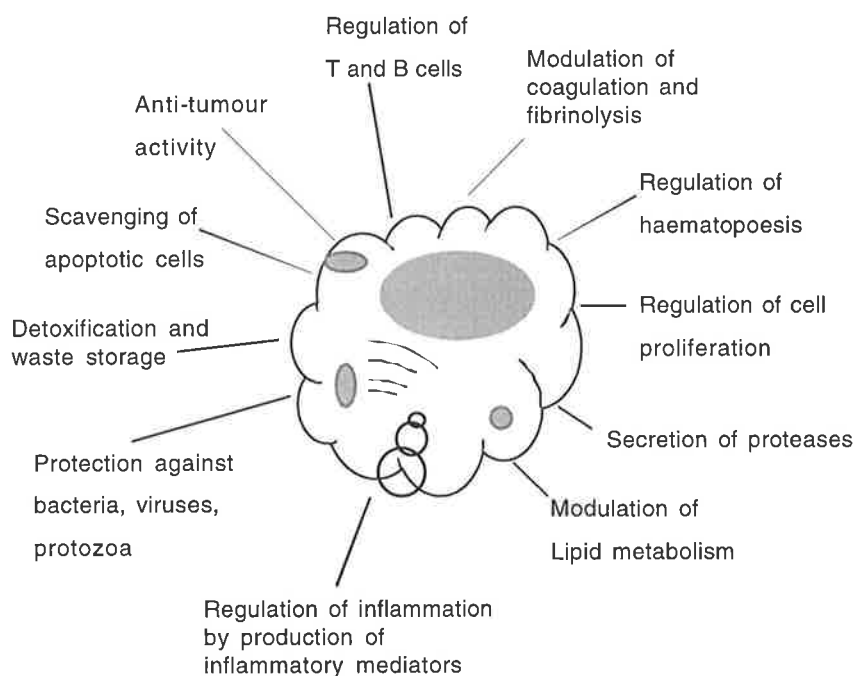
Diagram 1-4:   Synopsis of the diverse functions of monocytes and macrophages

Although macrophages are found in all tissues (peritoneum, liver, spleen, lymph nodes) they are particularly concentrated in the lung (alveolar macrophages and resident tissue macrophages). Macrophages are long-lived cells, which are specialized in providing immunity against bacteria,

viruses and protozoa capable of living within host cells. Resident macrophages are quiescent cells, however, they can be rapidly activated by inflammatory stimuli to become inflammatory macrophages. Phagocytosed organisms are killed by activated macrophages in various ways, through reactive oxygen intermediates, reactive nitrogen intermediates or by the secretion of enzymes, such as acid proteases, cathepsin, arginase and lysozyme. Apart from phagocytosis and killing of microorganisms and tumour cells, other important functions of activated macrophages are antigen presentation to MHC class II-restricted T-cells and B-cells and the production of cytokines, such as IL-1, TNF-α, IL-6, IL-8, IFN-γ and GM-CSF.

The secretory products of macrophages are essential contributions to the acute phase of the inflammatory process, macrophages are known to produce over one hundred defined molecular products. The ultrastructure of macrophages is indicative of their secretory ability. Mononuclear phagocytes possess abundant cytoplasm with large numbers of mitochondria, pinosomes, lysosomes and Golgi zones. The cytoplasm features a well-organized cytoskeleton of actin filaments and microtubules. With activation and maturation of the cell the number of these organelles increases.

1.1.1.2 Neutrophils

Neutrophils like monocytes originate from bone marrow stem cells. They are abundant in blood and normally not found in tissues except foci of inflammation. The number of neutrophils in the normal lung is about three times the circulating pool. The majority of these cells are marginated (travel near the vessel wall at reduced velocity) and located mainly in the alveolar capillaries (Gee et al., 1993). In contrast to macrophages, neutrophils are short-lived cells. Once they leave the bone marrow they survive only three to four days. Neutrophils are the early response unit of the innate immune system (Lloyd et al., 1992). They rapidly migrate to areas of infection, under the influence of chemoattractants such as IL-8 and enhanced by local upregulation of adhesion molecules (Kuijpers et al., 1992; Shall et al., 1994) (Diagram 1-5).
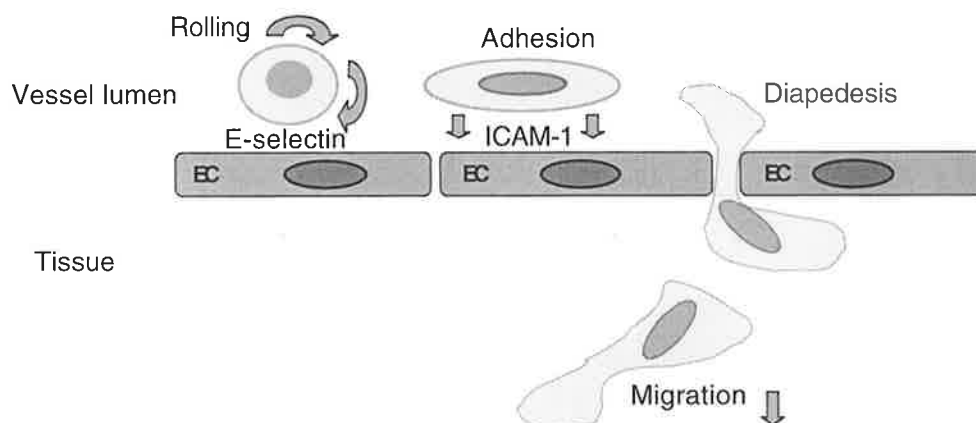
Diagram 1-5:   Simplified sketch of a neutrophil migrating through the endothelium (EC). Neutrophil surface CD15 (Sialyl-Lewis X) makes contact with E-selectin on endothelial cells (rolling). Engagement of neutrophil CD11b/CD18 with ICAM-1 leads to adhesion and diapedesis. Finally, the neutrophil migrates to the site of inflammation driven by chemotactic factors.

Neutrophils phagocytose and kill many different types of microorganisms. Like macrophages, neutrophils possess a number of bactericidal substances including proteases, acidifying enzymes, and reactive oxygen and nitrogen intermediates. They also secret anti-microbial peptides, such as bactericidal permeability-inducing peptide (BPI) and defensins. Other proteins of neutrophil origin such as lactoferrin, a chelator of iron and Vitamin B12, inhibit pathogens by means of binding these important microbial nutrients. All these antimicrobial agents are stored in cytoplasmatic granules (Sengelov et al., 1995). Upon activation of the cell the membrane enclosing those granules either fuses with the cell membrane (exocytosis) or with the membrane of a phagolysosome. Neutrophils possess two types of granules, azurophilic and specific, which can be mobilized independently (Wright et al., 1977). The contents of azurophilic and specific granules are summarized in Table 1-2.

Table 1-2:   Contents of neutrophil granules

Azurophilic granules	Specific granules
Myeloperoxidase (a haeme-protein)	Vitamin B12 binding proteins
Lysozyme and lysozyme proteins	Lysozyme
BPI (CAP 57)	Collagenase
Defensins (small, cysteine-rich proteins)	Gelatinase
Elastase	Lactoferrin
Cathepsin G	Plasminogen activator
Proteinase 3	Histaminase
Azurocidin (CAP 37)	Cytochrome b_{558}
β-glycerophosphatase	β_2 microglobulin
β-glucoronidase	Receptors
N-Acetyl-β-glucosaminidase	- fMLP-receptor
	- Laminin
α-Mannosidase	- CD11b/CD18 (CR3)
Cathepsin B	- Vitronectin
Cathepsin D	
CD14	

The notion that neutrophils cannot synthesize and secrete cytokines has been challenged. When stimulated with TNF-α, LPS or IL-1β, PMN produced significant quantities of IL-8 (Strieter et al., 1992). While the primary function of the neutrophil is microbial killing the cells can cause injury to host tissues in certain pathophysiological situations (Fukahori et al., 1997).

The generation of oxygen-derived reactive species (ODRS) is referred to as the 'respiratory burst'. This is one of the key mechanisms for intracellular killing of pathogens by leukocytes (Babior et al., 1972). The respiratory burst is a metabolic process of generating oxygen radicals by electron donation and enzymatic change, initiated by the intracellular nicotinamide-adenine dinucleotide (NADPH) oxidase complex. NADPH oxidase consists of two membrane-associated components,

cytochrome b_{558} and the flavocytochrome FAD, and four cytosolic components, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ (phox, phagocyte oxidase), and the GTP-binding protein rac 2 (Diagram 1-6).
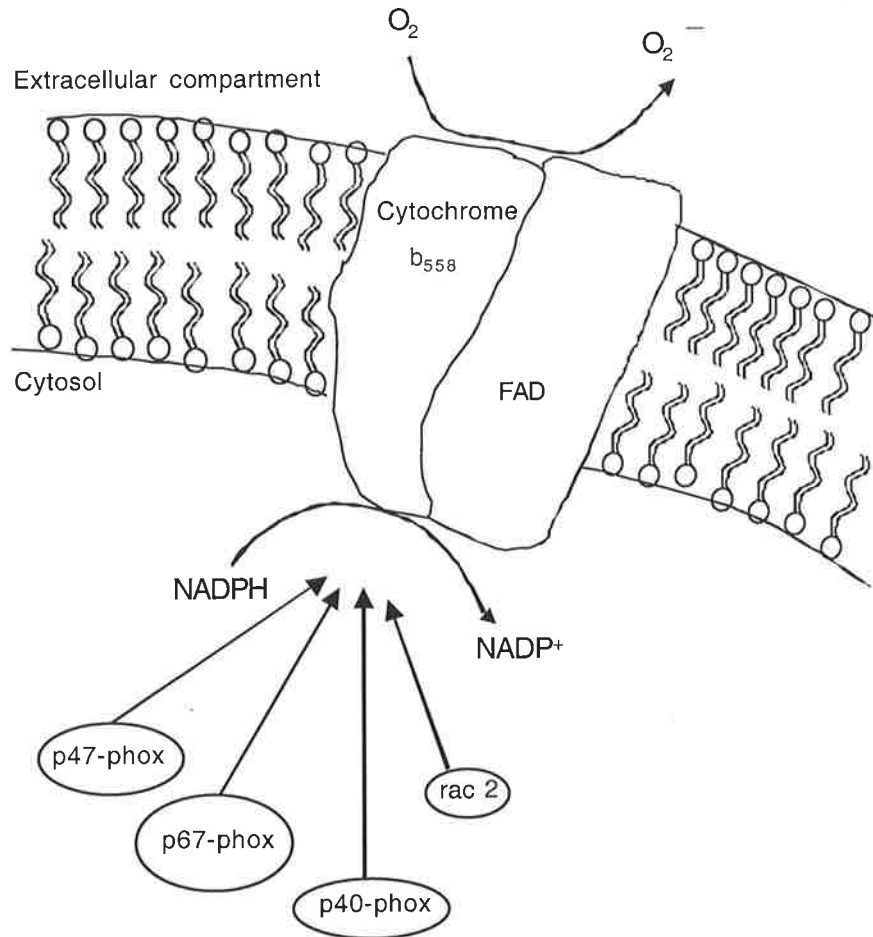
Diagram 1-6: Schematic representation of the NADPH oxidase complex. The heterodimeric flavoprotein (comprising cytochrome b_{558} and the flavocytochrome FAD) is found in the cell membrane. The 3 other components (p40-phox, p47-phox and p67-phox) reside in the cytosol if the cell is inactive, but get translocated to the flavoprotein complex upon cell activation. Activation of the complex also involves a small G protein, rac 2. (phox, phagocyte oxidase; NADP, nicotinamide-di-phosphate)

In response to activation of the neutrophil several kinases are able to phosphorylate the cytosolic components, which aggregate and translocate to the membrane. Rac 2 is also translocated to the membrane complex, which upon completion effects a conformational change in cytochrome b_{558}. This facilitates the extraction of electrons from NADPH and their transfer to oxygen via FAD (Hancock, 1997). The first product of this enzyme complex, generated within 30-60 minutes after cell activation, is superoxide (O_2^-), which is unstable and relatively impotent in terms of microbial killing. Superoxide dismutase (SOD) catalyzes the reaction of superoxide to hydrogen peroxide (H_2O_2), which is more cytotoxic than O_2^-. The myeloperoxidase–chloride system generates hypochlorous acid (HOCl), which is the most toxic product of the respiratory burst. Other ODRS are the hydroxyl radical (HO·) and singlet oxygen (O_2^-), both of which are not likely to be of major importance *in vivo*, because they are not formed in significant quantities and the kinetics of the reactions generating them

are relatively slow. The chemical reactions leading to the formation of ODRS are summarized in Table 1-3.

Table 1-3: The main reactions producing ODRS in the neutrophil respiratory burst

	NADPH oxidase	
$NADPH + 2O_2$	$\longrightarrow$	$2\,O_2^{-\cdot} + NADP^+ + H^+$
	SOD	
$2\,O_2^{-\cdot} + 2H^+$	$\longrightarrow$	$H_2O_2 + O_2$
	Myeloperoxidase	
$Cl^- + H_2O_2 + H^+$	$\longrightarrow$	$HOCl + H_2O$

ODRS cleave hydrogen atoms from polyunsaturated fatty acids and cleave oxygen-oxygen bonds. This lipid peroxidation destroys lipid bilayers of cell membranes and causes calcium influx in concentrations which are toxic to cells. Amongst other effects calcium activates proteases, which cleave actin-binding proteins, thus eliminating the plasma membrane anchor for the cytoskeleton, causing visible "blebbing" of the cell surfaces (Doelman et al., 1990).

1.1.2   Local tissue cells

Cells forming barriers, such as epithelial cells and endothelial cells play an important role in the inflammatory reaction. However, structural cells such as fibroblasts, smooth muscle cells and chondrocytes may also be involved in inflammation.

1.1.2.1 Epithelial cells

Epithelial cells, lining the skin, the gastro-intestinal tract, uro-genital tract and the respiratory system, are often the first point of encounter with bacterial organisms and can express adhesion molecules attracting leukocytes to the site of bacterial intrusion (Henderson et al., 1996). Epithelial cells can act as antigen presenting cells and secrete cytokines to recruit leukocytes or to activate leukocytes already at the area of inflammation (Simon et al., 1995). Finally, they can secrete substances directly harmful to bacteria, for example HCl in the stomach, cryptidins in the small intestine and surfactant, which has direct bactericidal effects in the airway.

Respiratory epithelial cells are unique since they have been reported to be the only nucleated cells which are hyposensitive or insensitive to bacterial lipopolysaccharide (LPS). However, some published data contradicts that observation (Table 1-4).

Table 1-4:   Reports on the effects of LPS on respiratory epithelial cells.

No effect on:	Reference	Claimed effect on:	Reference
MCP-1 production	Standiford et al., 1991		
IL-8 production	Standiford et al., 1990 Nakamura et al., 1991 Pugin et al., 1993	↑ IL-8 production	Keicho et al., 1997 Khair et al., 1994, 1996
ICAM-1 expression	Keicho et al., 1997	↑ ICAM-1 expression	Khair et al., 1994, 1996
IL-6 production	Crestani et al., 1994 Wang et al., 1999	↑ IL-6 production	Khair et al., 1994
IL-1β production	Wang et al., 1999		
TNF-α production	Wang et al., 1999	↑ TNF-α production	Khair et al., 1994

1.1.2.2 Endothelial cells

The endothelium is a specialized tissue forming a barrier between blood and extravascular compartments. Endothelial cells (EC) are thin cells with a large surface area, and their cytoplasm is rich in organelles and storage vesicles. They form a monolayer and are joined together by tight junctions. It has been estimated that the surface area of the entire vascular bed in an average-sized adult is about $1000m^2$ (Jaffe, 1985). Like epithelial cells vascular endothelial cells increase their adhesive properties upon activation, but also produce pro-coagulatory and vaso-active substances. When damaged endothelial cells can not maintain their tight junctions with neighbouring endothelial cells resulting in extravasation of plasma fluid and plasma proteins. If this occurs systemically rather than locally, the loss of intravascular volume can lead to hypotension, shock and death (eg. anaphylactic shock).

Endothelial cells interact with leukocytes via surface adhesion molecules, which they either express constituitively or synthesize and then express upon activation (Lo et al., 1989; Horwitz, 1997). The main adhesion molecules are intercellular adhesion molecule 1 (ICAM-1), E-selectin and vascular cell adheion molecule 1 (VCAM-1). These molecules have corresponding ligands on the leukocytes surface (Diagram 1-7).
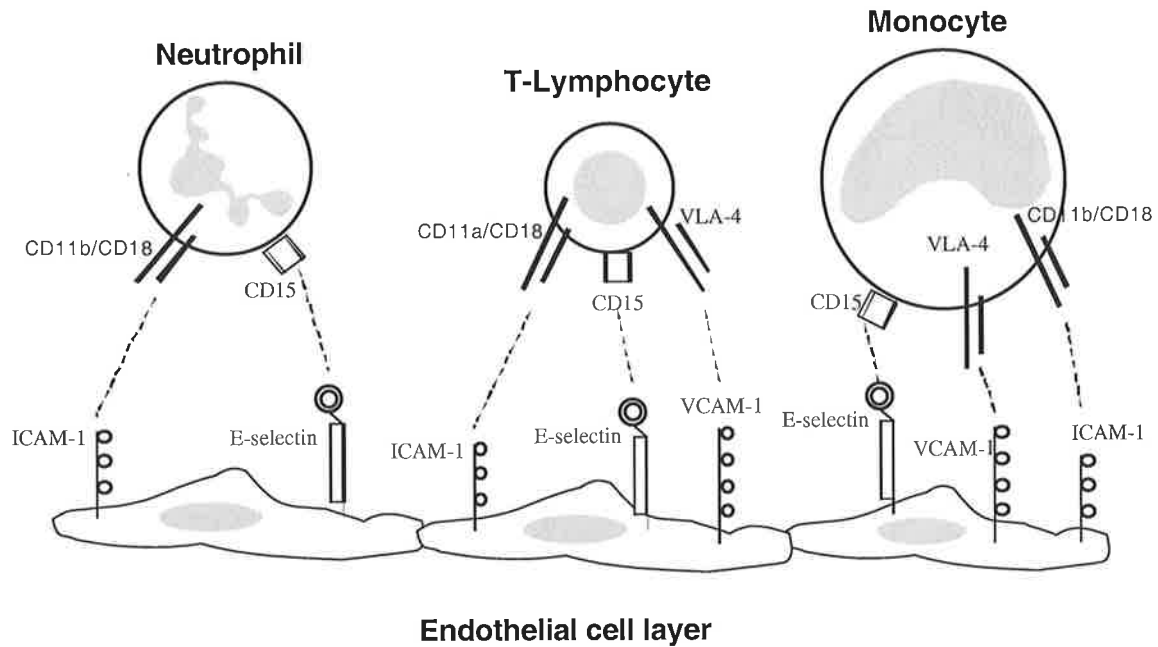
Diagram 1-7:   Adhesion molecules on endothelial cells and their interaction with different leukocytes. This sketch summarizes the interactions of the endothelial adhesion molecules E-selectin, ICAM-1, VCAM-1 with their corresponding ligands on neutrophils, T-lymphocytes and monocytes (adapted and modified from Springer, 1990 and Gamble et al., 1992).

1.2   MICROBIAL TOXINS (EXOGENOUS MEDIATORS) AND THEIR EFFECTS ON HOST CELLS

Microbes and their products can interact directly with and cause changes to host cells. Microbes bind to local cells such as epithelial or endothelial cells, leading to activation and often invasion of the target cell, in most instances resulting in target cell damage or death. Death or damage of barrier cells result in a breech of this barrier, facilitating further invasion by microorganisms. Exo- and endotoxins bind to specific receptors on target cells (Walmrath et al., 1994). Direct effects of toxins are exerted through receptors or by entering cells and interfering with vital cell functions such as protein synthesis. On the other hand toxins and microorganisms stimulate the release of mediators by host cells. For example, macrophages produce TNF-α and IL-1 when challenged by microbes or microbial toxins. TNF-α and IL-1 in turn lead to the production and release of further cytokines and to the activation of local and inflammatory cells. Because of the numerous ways of modifying the cytokine network bacterial mediators have been dubbed 'bacteriokines' (Henderson et al., 1995). Some of the bacterial interactions with the immune system are summarized in Table 1-5.

Table 1-5:   Interactions of bacteria with the cytokine network.

Type of interaction	Example
Cytokine synthesis	Bacteria or toxins activate macrophages to produce TNF-α and IL-1.
Functional molecular mimicry	Streptococcal pyrogenic exotoxin B (SPE B), a cystein protease mimicking ICE, leads to an IL-1β derivative which has biological activity.
Cytokine degradation	Bacterial proteases can digest and inactivate cytokines.
Suppression of cytokine synthesis or activity	The ADP-ribosylating toxin of Pseudomonas aeruginosa, Exotoxin A, inhibits the synthesis of IL-1α, IL-1β, TNF-α and INF-γ by phytohaemagglutinin- or *S. aureus* stimulated human monocytes.
Induction of cytokine receptor release and shedding	Proteases from *S. aureus*, *P. aeruginosa*, *L. monocytogenes* and *S. marcescens* have been shown to release the IL-6 receptor from human monocytes.
Increase of growth and proliferation of bacteria by cytokines	*M.tuberculosis* and *M. avium* can be proliferated with EGF via an EGF receptor. Intracellular growth of *M.tuberculosis* in macrophages is stimulated by TNF-α and TGF-β. *E.coli*, *Salmonella typhimurium* and *Shigella flexneri* bind TNF-α, and *E. coli* binds IL-1.

There are a large number of microbial toxins, which can have important actions on host cells during infections. The main toxins and their effects are listed in Table 1-6 below.

Table 1-6:   Microbial product and their effects on host cells.

Microbial product	Effect
Exotoxins	
Pseudomonas exotoxin (PE) A	PE-A is a 66 kD three-domain bacterial polypeptide toxin. Domain I is responsible for receptor binding, domain II plays a role in translocation and domain III contains an ADP-ribosylating activity. The toxin binds to a specific receptor on mammalian cells, a 300-400kDa glycoprotein, now identified to be the α_2-macroglobulin/low density lipoprotein receptor. Binding is followed by internalization via coated pits into the endosome compartment. PE hydrolyses NAD and transfers the ADPribose moiety to elongation factor 2 (EF-2). Modified this way EF-2 can no longer participate in the synthesis of new protein. The inactivation of EF-2 is lethal for the cell.
Diphteria toxin (DT)	DT acts very similar to PE. At non-toxic concentrations (less than 0.1-10µg/ml) it depresses immune responses, for example it reduces the production of TNFα, TNF-β, IFN-γ, IL-1α and IL-1β.
Haemolysin	Haemolysin of *E. coli* causes the release but not the synthesis of IL-1β.
Listeriolysin	Listeriolysin, a 58kDa thiol-activated haemolysin of *L. monocytogenes* causes IL-1 production by macrophages.
Toxin B	Toxin B (*Clostridium difficile*), a 269kDa protein increases IL-1, IL-6 and TNF-α synthesis in macrophages.
Pneumolysin	Pneumolysin of *S.pneumoniae* (pneumococcus) leads to IL-1β and TNF-α production. Pneumolysin is 200 times as potent as LPS. Shiga-like toxin type 1 induces release of IL-1a, IL-1β and TNF-α in macrophages.

Pertussis toxin Teichoic acids	Pertussis toxin of *B. pertussis* increases IL-4 production and upregulates IgE responses. These are endotoxins derived from the cell wall of gram-positive bacteria cause TNF-α, IL-1, IL-6 and IL-8 production in monocytes.
Lipoarabinomannan (LAM), lipomannan (LM) and phosphatidylinositol mannosides (PIM)	These substances are major cell wall constituents of mycobacteria, they make up up to 1.5% of their dry mass. These molecules are regarded as the mycobacterial equivalent of LPS. LAM induces human monocytes to produce TNF-α, IL-1α and β, IL-6, IL-8, IL-10 and GM-CSF. A cellular receptor has not been identified, however, LAM-induced effects on human monocytes are inhibited by anti-CD14 mab.
Heat shock proteins (HSP)	HSP are generated by bacteria in response to heat and induce cytokine production. A 65kDa HSP of *M. leprae* induced synthesis of mRNA for TNF-α in human monocytes. HSP can also cause release of IL-1α, IL-1β, IL-6, IL-8 and GM-CSF from macrophages.
Protein A	Protein A is a 42kDa surface protein from *S. aureus*, it mimics Fc and stimulates monocytes to release IL-1, IL-4, IL-6, TNF-α and IFN-γ.
Peptidoglycans	Peptidoglycans induce IL-1β, IL-6 and G-CSF production from monocytes. Anti CD14 mab are able to block peptidoglycan-induced cytokine release.
Lipid A-associated Proteins (LAP)	These proteins have IL-1-like activity and induce the acute phase reactant serum amyloid A (SAA). The active compound of LAP is about 17 kDa in size. LAP are several log orders more potent than LPS. A receptor for LAP has not yet been identified. LAP are very stable, resists boiling for 30 minutes and exposure to trypsin or pronase for 24 hours at 37^0C.
Porins	Porins originate from the cell wall of gram negative bacteria, they are ~100kDa in size, $2x10^5$ molecules per bacterium. They form trimers with a central pore of 1nm in diameter. Porins induce IL-1, IL-4, IL-6, IL-8, TNFα, GM-CSF and IFN-γ production by macrophages. PMN and endothelium are stimulated to release PAF.
Pili and Fimbriae	Pili and Fimbriae consist of a hydrophobic protein called pilin. It is found mainly on gram-negative bacteria with the exception of streptococci and actinomycetes. These mediators stimulate monocyte IL-1 and IL-6 production.
Surface-Associated Material (SAM)	Gentle saline extraction of a number of gram-positive bacteria yields a protein fraction which stimulates macrophages to produce IL-6, IL-1, IL-8 and TNF-α.
Lipoproteins (OspA and OspB)	These agents originate from *Borrelia burgdorferi*, a spirochaete, which is the causative agent of Lyme disease. OspA and OspB stimulate IL-6 and TNF-α production by macrophages. In multicentre double-blind randomised trials (21,241 human subjects) there was 68% efficacy in preventing Lyme disease after the initial two injections and 92% after a third "booster" injection.
Bacterial Superantigens	These are a large family of bacterial exotoxins which activate significant proportions of T-lymphocytes. They are mainly found in Staphylococci and Streptococci, but also in *Y. enterocolitica* and *P. aeruginosa*. Their structure is that of simple, nonglycosylated proteins. Superantigens bind directly to MHC class II molecules, bypassing antigen presenting cells. They then activate tyrosine kinase phosphorylation which increases TNF-α and IL-1 production. There are reports of synergism of superantigens with endotoxins. Interestingly, different superantigens have little if any amino acid sequence similarity, but share many three-dimensional structural features explaining their similar biological activity.
Proteases	Exotoxin B is a cysteine protease from *S. pyogenes* which produces a biologically active IL-1 like protein. Proteases of *P.aeruginosa* and *L. pneumophilia* inactivate IL-2, TNF-α and IFN-γ. The latter also cleaves CD14.
Bacterial lipopolysaccharide (LPS), endotoxin	LPS or endotoxin, is a major component of the cell wall of gram-negative bacteria with high biologic activity. LPS is dealt with in more detail in the following section.

1.3 BACTERIAL LIPOPOLYSACCHARIDE (LPS)

1.4.1 History

The beginnings of research into bacterial lipopolysaccharide date back to 1874, when Peter L Pannum, Professor of Physiology and Pathology at Kiel University (Germany) published his work characterizing a "putrid poison", partially purified from decaying flesh and fish. He described a substance presumed to be of bacterial origin, which was resistant to heat and fermentation, non-volatile and lethal to dogs at a dose of 12mg. The term 'endotoxin' was first used in 1892 by Richard Pfeiffer, a co-worker of Robert Koch in Berlin. Only in the late 1940s did Westphal and Luederitz manage to purify endotoxin using the hot phenol water extraction to yield a protein-free, highly toxic substance, which consisted only of carbohydrates, phosphorus and fatty acid. They named this substance lipopolysaccharide (Westphal et al., 1952). The mechanisms by which LPS was exerting its biological effects were not unravelled until recently (Ulevitch et al., 1994).

Lipopolysaccharide is found in the outer leaflet of the cell wall of gram negative organisms and enhances the flexibility of the bacterial cell membrane. It is probably important for bacterial replication and is thought to provide a survival advantage to the bacteria by making the cell membrane less permeable (Rietschel et al., 1992). Each bacterial cell possesses ~ 3-4 million LPS molecules, which cover ~75% of the cell wall surface.

Gram-negative bacteria and therefore LPS are ubiquitous in the environment. Especially high concentrations are encountered in food processing with health implications for the workforce in these industries (Rylander, 1995).

1.4.2 Definitions

Due to the significant biological activity of other products generated by the processing of bacteria and the increasing confusion caused by inappropriate usage of the terms 'endotoxin' and 'lipopolysaccharide', the following definitions have been adopted to clarify the results of scientific research. The term 'endotoxin' describes products of extraction procedures, which result in macromolecular complexes of LPS, protein and phospholipids. The term 'lipopolysaccharide' is reserved for purified bacterial extracts, which are reasonably free of detectable contaminants, particularly protein (Hitchcock et al., 1986).

1.4.3 Structure of LPS

Lipopolysaccharide has a low solubility and consists of a hydrophobic lipid region called Lipid A, and a hydrophilic polysaccharide domain (Henderson et al., 1996). The polysaccharide chain consists of core and O-antigen regions (Diagram 1-8A). The O-specific chain contains repeating oligosaccharides and up to five repeating sugar residues. Different bacteria display large variations in the O-antigen

structure, against which the major anti-LPS immune response is directed (Mayeux, 1997). The structure of the core region is considerably more conserved within gram-negative species (Ulevitch et al., 1995). Lipid A is a phosphorylated, multiply fatty acid-acylated glucosamine disaccharide that serves as the anchor of the entire structure in the outer membrane of the bacterial cell wall. Lipid A is the biologically active part of the molecule (Diagram 1-8B).

The three-dimensional structure of the lipid A molecule has been linked to its biological activity. Cylindrical shapes are non-toxic, cone-shaped lipid A possesses the highest toxicity (Rietschel et al., 1998). The apparent molecular weight of LPS is 500-1000 kD in micelles, a disaggregated single molecule is thought to be about 100 kD in size.
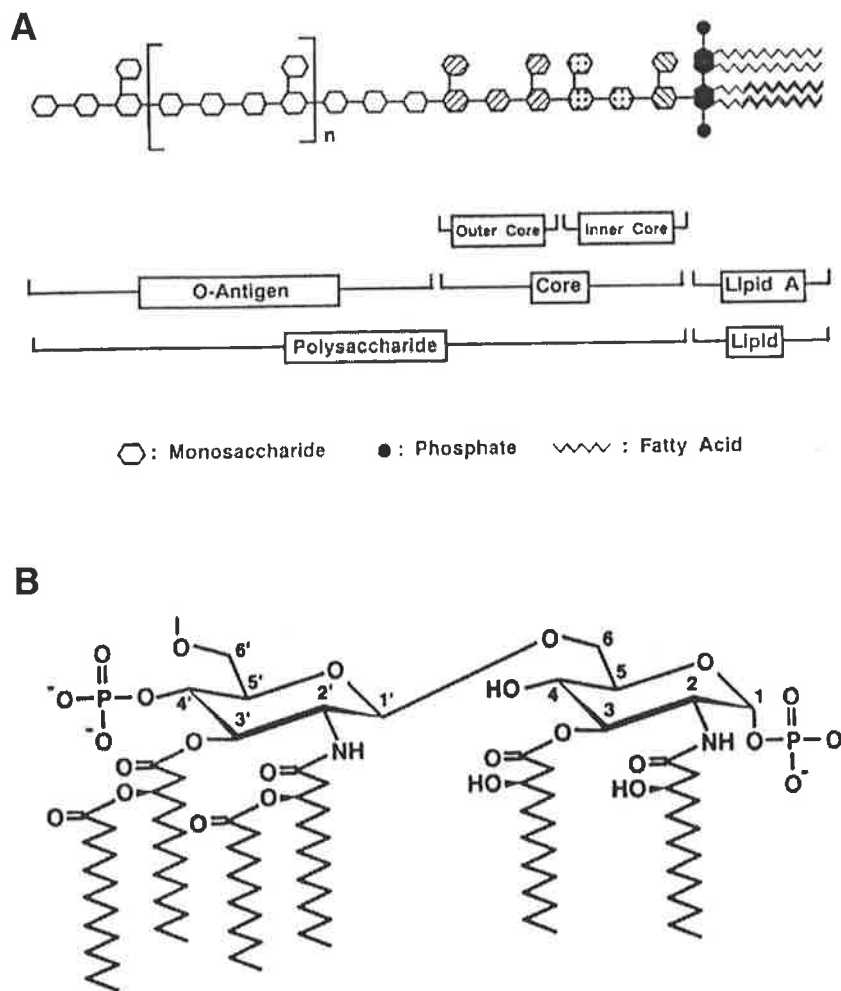
Diagram 1-8: Bacterial lipopolysaccharide. **A.** General chemical structure of LPS. **B.** Structure of the biologically active part of LPS, lipid A (*E. coli*). The term 'O-antigen' is derived from German "Ohne Hauch", no mist, to describe non-motile Proteus species. In contrast, 'H-antigen' , German "Hauch" was used to describe motile species of Proteus.

1.4.4   Biological activity

LPS is a potent stimulator of TNF-α production by macrophages and has also been shown to have the ability to prime macrophages and neutrophils for second agonists such as fMLP and TNF-α (Henderson et al., 1996). In animals injection of endotoxin causes rapid sequestration of neutrophils in the pulmonary vascular bed and shock-like symptoms (Haslett et al., 1987). LPS inhalation in healthy humans increases neutrophils, lymphocytes and fibronectin levels in bronchoalveolar lavage fluid (Sandström et al., 1992). Nearly all biological effects of LPS can be mimicked by purified Lipid A. The toxicity of LPS varies from non-toxic (*Rhodopseudomonas sphaeroides*) to highly toxic (Diphosphoryl lipid A of *E. coli*) and all LPS types compete equally for binding sites on the cell surface. This observation has led to the development of a range of therapeutic agents, ranging from natural non-toxic LPS to synthetic non-toxic LPS- or lipid A-like compounds. The three-dimensional structure of Lipid A seems to determine the toxicity of the LPS molecule, the more inverted the higher the endotoxin activity. Endotoxin activity is assessed by the amoebocyte *limulus*-lysate assay. LPS is relatively heat resistant, it withstands boiling for several hours.

Information on the *in vivo* effects of endotoxin in humans are derived from historical trials where LPS in high doses was used as an adjuvant agent in the treatment of malignancy (Brues et al., 1994). These large doses (15,000ng/kg) unfortunately caused shock and disseminated intravascular coagulation. More recently endotoxin was administered to healthy human volunteers in low doses (Suffreidini et al., 1989; Martich et al., 1991). In those experiments small amounts of endotoxin (4ng/kg) caused an increased cytokine production, increased fibrinolytic activity, a decrease in systemic vascular resistance and increased capillary permeability (Taveira da Silva et al., 1993; van Deventer et al., 1990).

1.4.5   LPS serum levels

In healthy human volunteers serum levels of LPS are low, about 10pg/ml (Martin et al., 1997). In meningococcal sepsis LPS serum levels range from <25pg/ml to 19ng/ml and correlate with organ failure and death. Others have reported levels in septic patients to be between 100-500pg/ml (Martin et al., 1994). A strong correlation has been described between detectability of significant amounts of circulating LPS and outcome in septic patients, leading to the recommendation to introduce a rapid endotoxin detection kit as a prognostic tool (Kollef et al., 1997).

1.4.6   Serum factors

In aqueous solutions LPS forms aggregate micelles or membranes with sheet-like structures of 94Å thickness because of its lipid properties. LPS is reported to bind to a wide range of serum proteins, which break up aggregate structures and aid transport of the molecule (Table 1-7).

Table 1- 7: **Serum proteins reported to bind to LPS**

Serum protein	Reference
Lipoprotein binding protein (LBP)	Tobias et al., 1988
Soluble CD14 (sCD14)	Schütt et al., 1992
Free haemoglobin	Roth et al., 1994
Albumin	Dziarski, 1994
Bactericidal/Permeability-inducing protein (BPI)	Dentener et al., 1993
Lactoferrin	Appelmelk et al., 1994
Lipoproteins	Ulevitch et al., 1979
Mac-2-BP	Yu et al., 1995
Septin	Wright et al., 1992

Some of these proteins interact non-specifically with LPS, yet some are facilitating LPS binding to cells or the removal of LPS from the circulation. LBP and sCD14 are recognized as the main facilitators of LPS-induced cell activation. LBP is a member of a group of homologous lipid binding proteins and it binds to LPS in serum via its Lipid A region. LBP then transfers LPS to CD14 (Ulevitch et al., 1999).

LPS can activate cells in the absence of serum. However, 100- to 1000-fold higher concentrations are required to achieve a comparable effect (Von Asmuth et al., 1993).

1.4.7 Cellular receptors for LPS

During the past two decades theories had been put forward that LPS activates cells simply by intercalating into the lipid pool of the cell membrane without using any receptor. However, compelling evidence such as blocking LPS-induced cell activation by monoclonal antibodies against surface structures or abrogating LPS responses by pre-treatment of cells with trypsin and competitive inhibition of LPS action by non-toxic LPS molecules, point to the existence of a specific receptor or receptor-system. Interestingly, such a receptor has not been unequivocally identified to date. The following molecules have been proposed as LPS receptors (Table 1-8).

Table 1-8: **Putative cellular receptors for LPS**

Membrane protein	Evidence for signalling	Reference
Albumin	No	Dziarski, 1994
BPI (neutrophil surface)	No	Weersink et al., 1993

CD11a/CD18	Yes	Ingalls et al., 1998
CD11b/CD18	Yes	Wright et al.,1989; Lo et al., 1989
CD11c/CD18	Yes	Ingalls et al., 1995
80kD membrane protein	Not examined	Schletter et al., 1995
95kDa Scavenger receptor	No	Hampton et al., 1991
CD14	Yes	Wright et al., 1990
hTLR2	Yes	Kirschning et al., 1998, Yang et al., 1998
hTLR4	Yes	Medzhitov et al., 1997, Poltorak et al., 1998
Moesin	Yes	Tohme et al., 1999

1.4.8   Summary of the current concept of LPS-induced cell activation

The currently accepted model of LPS-induced activation of cells is that LPS first forms a complex with LBP in serum and this complex then binds to either mCD14 or to sCD14. CD14 is believed to act as a lipid transfer protein and pass LPS onto a transmembrane protein capable of initiating signal transduction.

1.4.9   Fate of LPS in the circulation

LPS is cleared from the circulation via a pathway distinct from that, which leads to signalling (Gegner et al., 1995; Vasselon et al., 1999). When injected into rats the liver was the found to be the prime site of accumulation of LPS, where it was taken up by Kupffer cells, neutrophils and hepatocytes (Freudenberg et al., 1984). Inhaled LPS is processed mainly by alveolar macrophages (Keller et al., 1991). Once taken up by cells LPS is rapidly transported to the Golgi apparatus (Thieblemont et al., 1999). Macrophages are the most efficient LPS-detoxifying cells (Rutenburg et al., 1960), although biologically active LPS has been found in macrophages several days after the initial exposure (Duncan et al., 1984). Neutrophils also detoxify LPS, deacylation by acyloxyacyl hydrolase takes place in the acidic intracellular compartment (Luchi et al., 1993). After administration to rats it took several weeks for the LPS to be cleared completely, it was excreted mainly via the faeces (Freudenberg et al., 1984).

1.5   ENDOGENOUS MEDIATORS OF INFLAMMATION AND PRO-INFLAMMATORY CYTOKINES.

The term 'cytokine' originates from early observations of the phenomenon of chemotaxis. Cytokines are small proteins mediating signals between cells. These signals can affect the cell that generates the cytokine (autocrine), neighbouring cells (paracrine) or distant cells (endocrine). Apart from inducing migration, cytokines control the function, growth and survival of other cells. The hallmark of cytokines is pleiotropy (Greek: πλειος, full of; τροπος, direction), which must not be mistaken for

redundancy. Although most cells are capable of producing cytokines they are predominantly secreted by monocytes/macrophages upon contact with bacterial products. In this thesis we focused on proinflammatory cytokines only, including IL-1β, IFN-γ, IL-6. However, the emphasis of our studies was with the most pleiotropic of all cytokines, TNF-α.

1.5.1   Tumour necrosis factor alpha (TNF-α)

The existence of this factor was first presumed over a century ago by William Coley, who observed, that sarcoma patients went into remission after suffering incidental gram negative infections (Coley, 1891). TNF-α was isolated in 1975 by Carswell and was later found to be identical to cachectin, a substance causing nausea, loss of appetite and wasting, initially thought to be a different mediator (Carswell et al., 1975; Beutler et al., 1985). TNF-α is a 17,356 Dalton protein, which oligomerizes to form trimers (Diagram 1-9).
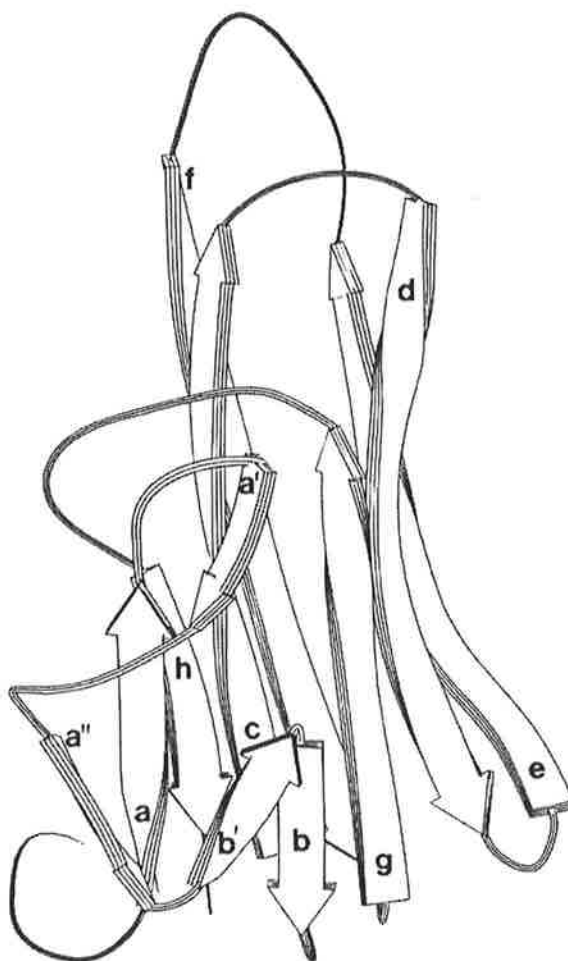
Diagram 1-9:   Structure of TNF-α. Schematic image of the three-dimensional structure of a TNF monomer. Individual structural elements are labeled according to Eck et al., 1989. (Residues 12-18, **a**; 26-30, **a'**; 36-39, **a"**; 41-45, **b'**; 47-51, **b**; 56-67, **c**; 72-84, **d**, 88-101, **e**; 112-125, **f**; 129-138, **g**; 150-155, **h**)

The gene for TNF-α is located on the short arm of chromosome 6 near the HLA-B locus in the MHC-region. Although macrophages are the major source of TNF-α, all nucleated cells can produce it. There are two cellular receptors for TNF-α, TNF-R I (p55, 55kD) and TNF-R II (p75, 75kD) (Aggarwal et al., 1985). TNF-α is the most pleiotropic cytokine known and it has a wide range of biological actions as listed in Table 1-9.

Table 1-9: Biological effects of TNF-α.

Supression of anticoagulant properties of endothelium

Increase in adhesive properties of leukocytes

Upregulation of endothelial adhesion molecules

Stimulation of endothelial cell proliferation

Stimulation in dermal and pulmonary fibroblast proliferation

Induction of chondrocytes to increase collagenase and PGE2 production

Stimulation of osteoclasts (bone resorption)

Stimulation of the neutrophil respiratory burst

Promotion of insulin resistance

Inhibition of lipoprotein lipase and lipid storage in fat tissue

Inhibition of erythroid progenitor cells in the bone marrow

Causes hypotension and shock

Suppression of myocardial function

Causes central anorexia by direct effects in the brainstem

Causes fever, myalgias and fatigue

Stimulation of hepatocytes to increase production of acute phase proteins

Reduction in iron incorporation, contributing to the anaemia of chronic disease

Recently the role of TNF-α in the pathophysiology of chronic congestive cardiac failure and the preliminary success of TNF antagonism has generated considerable interest (Francis, 1999).

1.5.2 Interleukin-1 (IL-1)

The interleukin-1 family consists of three structurally related polypeptides. The first two are IL-1α and IL-1β, the third is interleukin-1-receptor antagonist. The gene encoding the interleukin-1 family is located on chromosome 2q. There are two cellular receptors for the IL-1 family, however, only IL-RI is known to possess signalling capacity. Like TNF-α, IL-1β is produced by monocytes and macrophages upon stimulation by bacterial products. IL-1 in turn stimulates macrophages to produce and release IL-8 (Bittleman et al., 1995). IL-1α is produced by epithelial cells. IL-1α and IL-β increase the adhesive properties of vascular endothelium, epithelial cells and leukocytes. Like TNF-α they induce sleep, cachexia, fever, acute-phase protein synthesis in the liver, bone resorption and

muscle proteolysis (Marsh et al., 1996). Interleukin-1-receptor antagonist is a specific inhibitor of IL-1 activity that acts by blocking the binding of IL-1 to its cell surface receptors and hence can mitigate the pro-inflammatory effects of IL-1.

1.5.3   IL-6

While TNF-α and IL-1 are produced after macrophages come in contact with bacterial products IL-6 and IL-8 are synthesized upon stimulation of macrophages by TNF-α. Amongst their many functions they are chemotactic for neutrophils, causing the accumulation of this cell type at the site of inflammation.

IL-6 has multiple biological activities against all cell types, in part overlapping with IL-1. It stimulates neutrophils to degranulate and primes neutrophils for an enhanced respiratory burst, although unable to elicit the respiratory burst by itself (Borish, 1989). IL-6 seems to play a role in pulmonary disease and lung damage, its activity was many fold increased in subjects with self-limited ARDS as compared to patients with lung infection without secquelae. The activity was higher again in children who developed BPD (Bagchi, 1994) IL-6 is the major stimulant for hepatocytes to synthesize acute-phase proteins (Xing et al, 1998). It also stimulates immune globulin production and B cell growth (Marsh et al., 1996).

1.5.4   Interferon gamma (IFN-γ)

IFN-γ is a monomeric glycoprotein and is chiefly produced by inflammatory T cells. It influences the growth, differentiation and activity of NK cells, T cells and B cells. IFN-γ increases expression of MHC class I molecules on many cell types and MHC class II molecules on some. IFN-γ stimulates macrophages to produce TNF-α and IL-1 and induces macrophage NO synthase, enhancing the ability of this cell type to kill pathogens. It also elicits the respiratory burst in neutrophils and causes lysosomal enzyme release (Kowanko et al., 1987). IFN-γ has been reported to synergize with other cytokines such as TNF-α. These effects of IFN-γ lead to leukocytes being more effective in inactivating gram-negative and gram-positive bacteria (Murray et al., 1988).

1.5.5   Granulocyte-macrophage colony-stimulating factor (GM-CSF)

GM-CSF is a haematopoetic growth factor. It is produced by mononuclear phagocytes and stimulated T-lymphocytes. It is a glycoprotein of a MW of ~ 22kD, which stimulates not only neutrophils and monocytes but also eosinophils. GM-CSF prolongs survival of these target cells, increases protein

synthesis, chemotaxis and primes cells for degranulation, production of AA and LTB_4 in response to other agonists.

1.6   INTRACELLULAR SIGNALLING

1.6.1   Introduction

Cells react to diverse stimuli with a range of different responses (proliferation, activation, inhibition or death). Cell signalling is concerned with the events taking place inside cells to external stimuli. In the past years there has been an exponential increase in research in this area (Krebs, 1994; Kyriakis et al., 1996). Stimuli causing cell signalling maybe non-specific (UV, heat, radiation, hyperosmolarity, H_2O_2) or specific (cytokines, toxins, antibodies). Specific activation of cells is mediated through cell surface receptors. Engagement of such receptors by their ligand leads to changes of conformation or association in the intracellular domain of the receptor, and eventually to protein phosphorylation. This activates enzymes (kinases), which then in turn become active themselves, being able to phosphorylate other kinases in a cascade manner. Ultimately these signalling cascades activate transcription factors in the cell nucleus. These factors bind to specific promoter regions on DNA, which initiates the process of gene transcription leading to protein synthesis. Research into signalling is complex and care has to be taken when interpreting results from different species and different cell types. Common methods to investigate signalling pathways are summarized in Table 1-10.

Table 1-10: Techniques utilized in signalling research

Receptors

- blocking (antagonist, antibody)
- activation (activating antibody)
- cleaving (enzymes)
- bypassing (PMA)
- genetic ('knock-outs')
- artificial insertion (transfection)

Signalling molecules

- blocking (antagonists)
- genetic (overexpression)
- genetic (transfection of mutants)
- genetic (knock-outs)
- measuring activation
- measuring degradation

Nuclear factors/Transcription factors

- activation
- translocation into the nucleus
- binding to gene loci
- genetic (knock-outs)

Genes

- estimation of mRNA
- measuring the protein product

In the following section the signalling pathways of the proinflammatory cytokines TNF-α, IFN-γ and IL-1β and also of bacterial lipopolysaccharide (LPS) are reviewed.

1.6.2   Signalling through the TNF-α receptor

TNF receptors belong to the super family of the nerve growth factor receptors and are expressed on the membranes of virtually all somatic cell types. Their numbers are typically low, from a few hundred to a few thousand per cell. There are two receptors, TNF-RI (55kD) and TNF-RII (75kD). The TNF receptors have four components, an intracellular domain, a single transmembrane segment, an extracellular cystein-rich domain and a hydrophobic single peptide. From studies with agonistic antibodies it is apparent that cross-linking of TNF-RI is required for cell activation. There is evidence that the TNF-RI is the main mediator of cell activation and TNF-RII plays an amplifying role (Leeuwenberg et al., 1995). However, expression of these two receptor types is independently regulated and both receptors mediate activation of the transcription factor NF-κB (Hohmann et al., 1990).

In mice not expressing TNF-RI (p55-/-) the TNF-induced expression of the endothelial adhesion molecules E-selectin, ICAM-1 and VCAM-1 is abolished, whereas in mice not expressing TNF-RII (p75-/-) the TNF-α-induced upregulation of these adhesion molecules was not affected (Neumann et al., 1996). When endothelial cells were activated with either TNF-RI or TNF-RII-specific agonistic antibodies, only stimulating TNF-RI upregulates adhesion molecules (Mackay et al., 1993). The binding species of the TNF receptors is a homotrimer of TNF-α.

Upon binding the TNF-α trimer both TNF receptors become internalized and probably degraded, since no receptor recycling could be demonstrated thus far. TNF-induced trimerization of TNF-RI triggers an association with TNF-RI-associated death domain (TRADD), which recruits receptor-interacting protein (RIP), a death domain-containing serine/threonine kinase (Yuasa et al., 1998), and TNF receptor-associated factor 2 (TRAF2) to form a TRADD-RIP-TRAF2 complex (Diagram 1-11). The recruitment of the Fas-associated death domain (FADD), which leads to apoptosis, is not included in Diagram 1-10 and will not be discussed here. TRAF2 and RIP activate downstream signalling pathways, which are described in detail below.
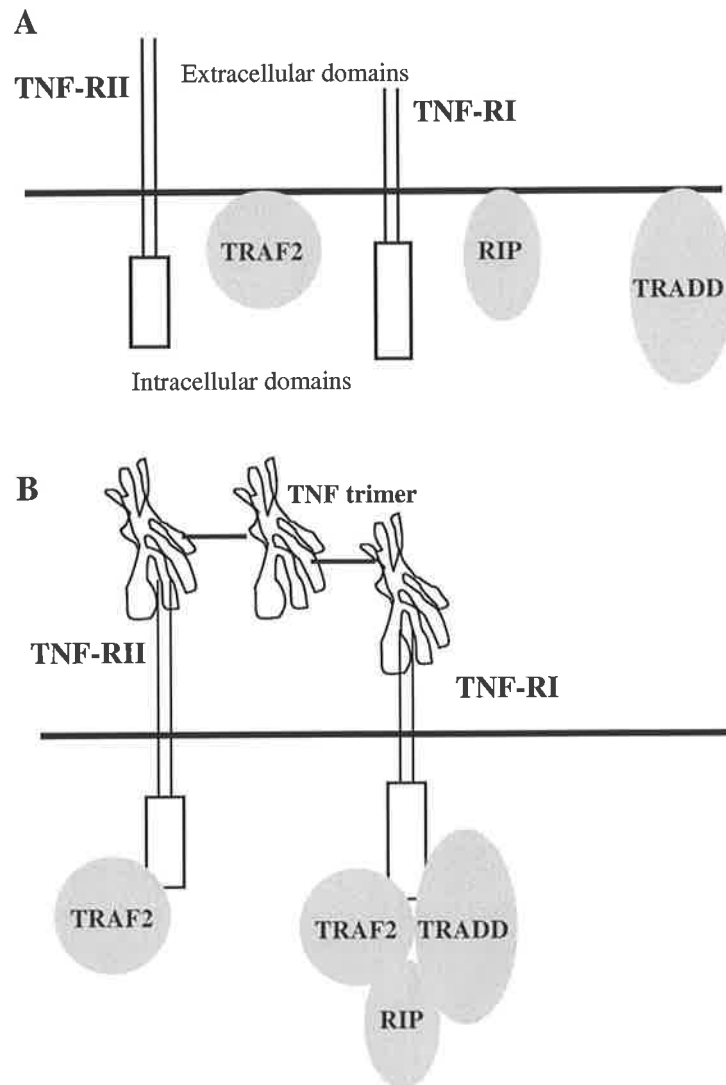
Diagram 1-10:   TNF receptor signalling. TNF-R, TNF receptor; TRAF2, TNF receptor-associated factor 2; TRADD, TNF receptor-associated death domain; RIP, receptor-interacting protein. **A.** Without TNF-α engaging its receptors TRAF2, RIP and TRADD are dissociated. **B.** When TNF-α binds to the TNFR a TRADD-TRAF2-RIP complex is formed at TNF-RI, whereas TNF-RII can bind TRAF2 without TRADD. TNF-RII is thought to enhance signalling by TNF-RI.

1.6.3   Signalling by IFN-γ

The receptor for IFN-γ consists of two heterodimeric subunits, IFN-γRα and IFN-γRβ. These subunits are associated with Janus kinases, JAK1 and JAK2 respectively. IFN-γ binding results in receptor dimerization/oligomerization and phosphorylation of JAK1 and JAK2. In turn these two Janus kinases phosphorylate the intracellular domain of IFN-γRα, recruit signal transducer and activator of transcription-1 (STAT1) and phosphorylate it. STAT1 needs also to be serine-phosphorylated in position S727. This is effected by another kinase, proline-rich tyrosine kinase 2 (Pyk2), which is recruited and activated by JAK2 (Takaoka et al., 1999).

In addition, the MAP kinase extracellular signal-regulated protein kinase (ERK) can also phosphorylate STAT1 *in vitro*. However, IFN-γ does not seem to activate the MAP kinase pathways (Han et al., 1993). Phosphorylated STAT1 dimerizes and translocates into the nucleus, where it binds to the γ-activation site (GAS) of IFN-γ-inducible genes (Darnell et al., 1994; Hussain et al., 1999) (Diagram 1-11).
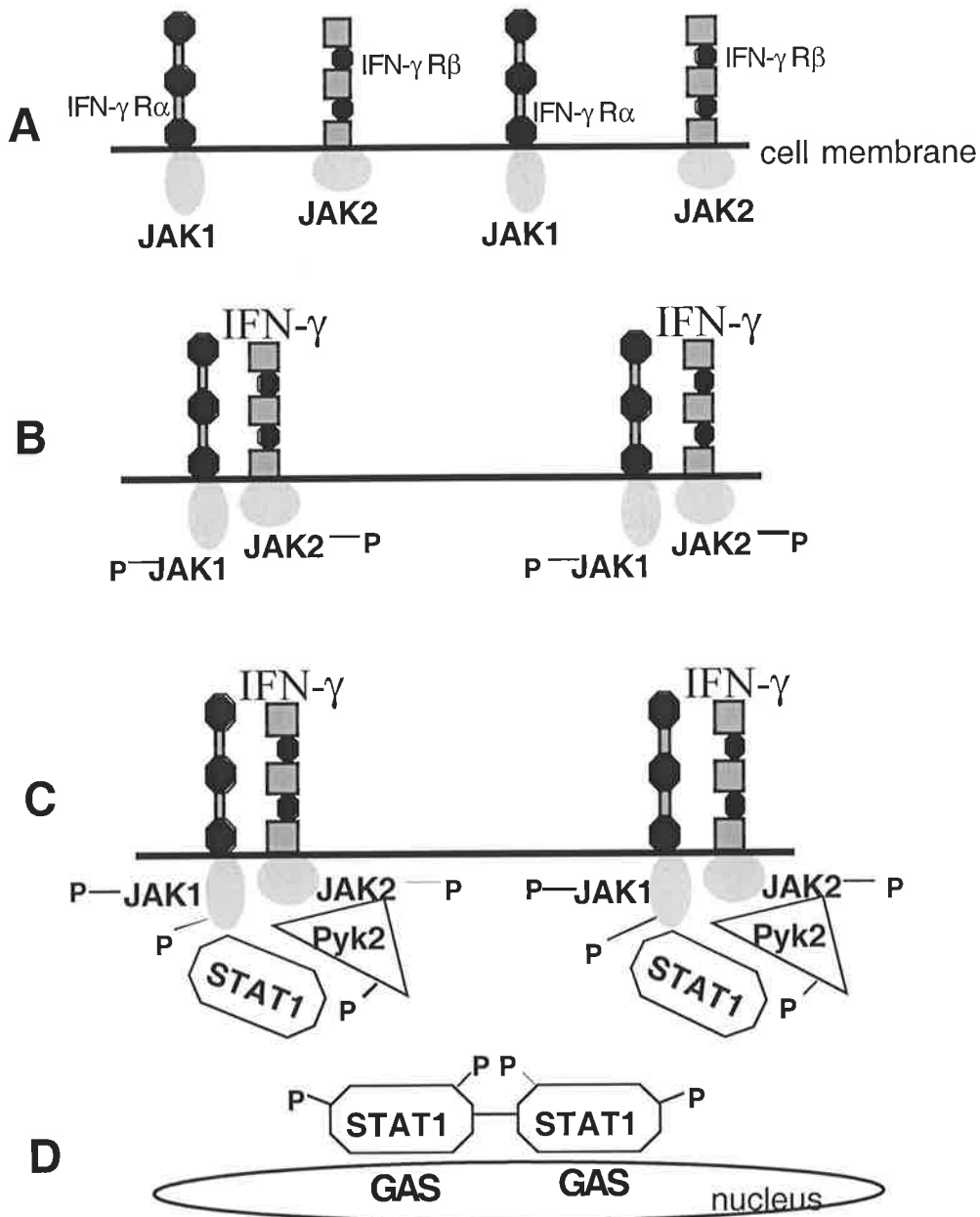
Diagram 1-11:   Signalling through the IFN-γ receptor. Binding of IFN-γ to its receptor causes dimerization of the α and β subunits and phosphorylation of JAK1 and JAK2, which phosphorylate the α-subunit and recruit STAT1. Phosphorylated STAT1 is also serine-phosphorylated in the S727 position by Pyk2, dimerizes and binds to GAS in the nucleus. JAK, janus kinase; STAT, signal transducer and activator of transcription; Pyk2, proline-rich tyrosine kinase; GAS, γ activation site.

1.6.4 Signalling by IL-1

Both, IL-1β and IL-1α signal through the IL-1 receptor (IL-1R), a member of the immunoglobulin superfamily of receptors. IL-1 binding to IL-R induces the formation of a receptor complex that includes the IL-1 accessory protein (IL-1AcP). IL-1AcP does not bind to the IL-1 receptor directly, but is essential for IL-1 signalling (O'Neill et al., 1998). Myeloid differentiation-factor 88 (MyD88), a 35kD adapter protein, is then recruited to the complex (Thomas et al., 1999). MyD88 possesses three functional domains, one to associate with the intracellular IL-1 receptor domain, one with interleukin-1 accessory protein (IL-1AcP) and one with interleukin-1 receptor-associated kinase (IRAK) (Cao et al., 1996a).

Studies with MyD88-deficient mice confirmed that this molecule is essential for signalling through the IL-1 receptor (Adachi et al., 1998; Kawai et al., 1999). IRAK, which is the vertebrate homologue of the *Drosophila* protein kinase Pelle, is phosphorylated and interacts with the TNF receptor-associated factor-6 (TRAF6) (Cao et al., 1996b). TRAF6, like TRAF2, activates downstream signalling pathways (Diagram 1-12)..
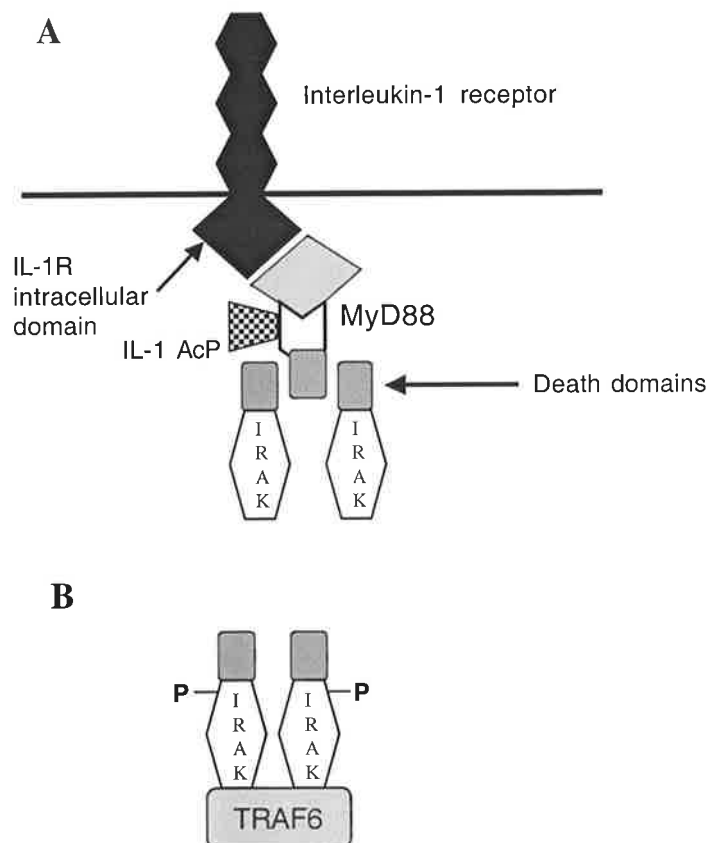
Diagram 1-12: The interleukin-1 receptor. Myeloid differentiation factor 88 (MyD88) has 3 interaction sites, one with the intracellular domain of the IL-1 receptor, one with IL-1 accessory protein (IL-1AcP) and one with the death domain of interleukin-1 receptor-associated kinase (IRAK). After phosphorylation IRAK dissociates from the receptor complex, dimerizes and interacts with TRAF6.

1.6.5 Signalling by LPS

Investigation of the signalling mechanisms utilized by bacterial lipopolysaccharide (LPS) via CD14 have been hampered by the fact that no cell membrane signalling receptor had been identified until 1998, although the evidence had pointed to the existence of a transmembrane receptor for a long time. Human equivalents of the Toll receptors of the fruit fly, human Toll-like receptor-2 and 4 (hTLR-2 and hTLR-4), have now been shown to mediate signalling by LPS (Medzhitov et al., 1997; Kirschning et al., 1998; Poltorak et al., 1998; Yang et al., 1998). These hTLRs belong to a family of five human Toll analogues, all related to the IL-1 family of receptors (Rock et al., 1998). Human Toll-like receptors utilize the signalling apparatus of the IL-1 receptor (Zhang et al., 1999). While the naturally occurring interleukin-1 receptor antagonist (IL-1RA) blocks cell activation by IL-1, it does not block LPS-induced signalling, proving that the extracellular domain of hTLR is different from IL-1R.

Upon engagement of the hTLR by LPS the adapter protein MyD88 is recruited to the intracellular domain of the receptor. Like in signalling by IL-1 IL-1AcP and IRAK associate with MyD88 resulting in phosphorylation of IRAK (Diagram 1-13). Transfection studies have proven the involvement of these molecules in LPS signalling. Dominant negative mutants of MyD88, IRAK or TRAF6 inhibited LPS-induced NF-κB luciferase activity (Zhang et al., 1999).
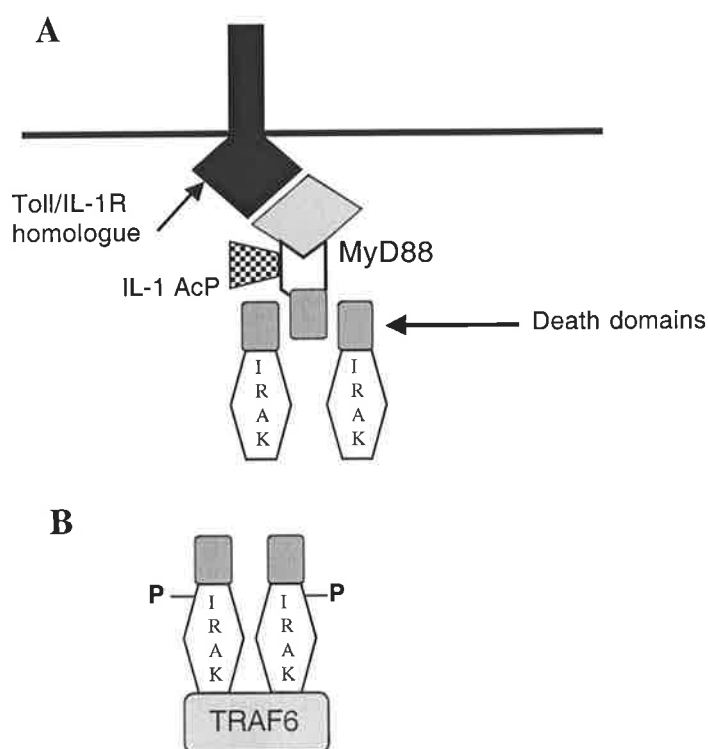
Diagram 1-13 : LPS signalling through the human Toll-like receptor. The hTLRs are thought to share a homologue intracellular domain with the IL-1 receptor. The adapter protein MyD88 binds to this domain and IL-1 accessory protein (IL-1 AcP), recruiting unphosphorylated IRAK to the complex. Phosphorylated IRAK dissociates from the complex, dimerizes and binds and phosphorylates TRAF6.

1.6.6   The nuclear factor-κB (NF-κB) pathway

NF-κB is an ubiquitous transcription factor and plays a crucial role in the regulation of numerous genes, particularly those involved in cell death and the inflammatory response (Schulze-Osthoff et al., 1997). Proinflammatory cytokines and LPS rapidly activate NF-κB. However, other activators such as ceramide and sphingomyelinase have been described (Baeuerle et al., 1994). NF-κB consists of two subunits, p65 (also known as Rel A) and p50. While p65 is the dominant, transcriptionally active subunit p50 serves merely as a DNA-binding element (Schmitz et al., 1995). In resting cells the NF-κB p65/p50 heterodimer is located in the cytosol and is associated with its natural inhibitor, IκBα. Activation is brought about by the phosphorylation of IκBα by the IκB-kinases alpha and beta (IKKα, IKKβ) (Bennett et al., 1996). IκBα dissociates from the p65/p50 subunits and undergoes proteolytic degradation (Traenckner et al., 1995). Therefore subsequent inhibition of NF-κB requires new synthesis of IκBα (Baeuerle et al., 1994). NF-κB with its DNA-binding site exposed by the dissociation of IκBα translocates into the nucleus and binds to the gene promoter region (Diagram 1-14).
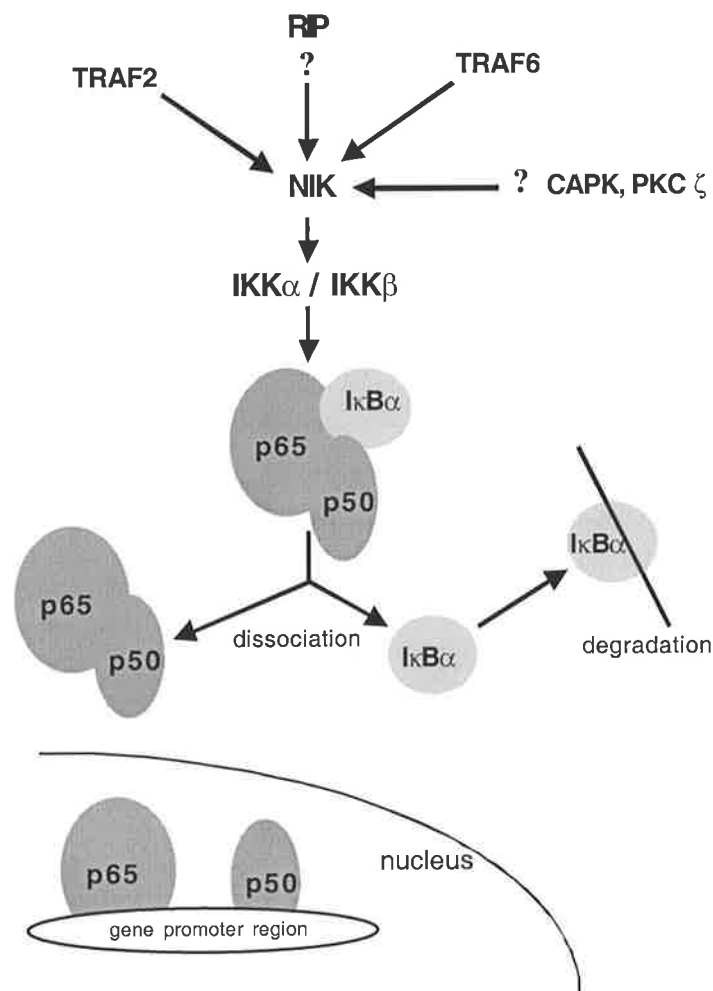
Diagram 1-14:   The NF-κB pathway. NF-κB-inducing kinase (NIK) activates IκB kinases (IKK), which phosphorylate IκB, which then dissociates from the p65/p50 heterodimer and undergoes degradation. The p65 and p50 subunits translocate into the nucleus and bind to DNA gene promoter regions with p65 playing a dominant role. CAPK, ceramide-activated protein kinase.

The activity of IKKα and IKKβ is elevated following cell activation by proinflammatory stimuli including TNF-α, IL-1β and LPS and is thought to be mediated by NF-κB-inducing kinase (NIK) (Bennett et al., 1996; Malinin et al., 1997). Overexpression of NIK leads to activation of NF-κB in the absence of a stimulus, and inserting a kinase-dead mutant of NIK inhibits TNF-α and IL-1β-induced activation of NF-kB (Malinin et al., 1997; Schulze-Osthoff et al., 1997).

Although it is well recognized which stimuli cause activation of NF-κB it is not entirely resolved how the pathways initiated by these mediators connect to the NF-κB system. It is most likely that molecules like the TRAF family directly activate NIK. For the TNF-α receptor-mediated pathway it has been shown that TRAF2 and RIP, either in parallel or in concert bind and activate NIK (Pober, 1998). Also, overexpression of TRAF2 activates NF-κB (Min et al., 1997). N-terminally truncated TRAF2 abrogated TNF-α-induced activation of NF-κB (Natoli et al., 1996; Min et al., 1997). However, in these experiments the activity of NIK itself was not examined. With respect to IL-1 and LPS-induced cell activation it has been demonstrated that TRAF6 directly interacts with NIK (Cao et al., 1996b; Regnier et al., 1997; O'Neill et al., 1998). Interestingly, the dissociation of IκBα from the NF-κB heterodimer is not sufficient for the activation of NF-κB. Inhibition of proteolysis of IκBα inhibited TNF-α-induced upregulation of endothelial cell adhesion molecules (Chen et al., 1995).

1.6.7   The mitogen-activated protein kinases (MAP kinases)

In recent years a family of mammalian homologues of yeast mitogen-activated kinases has been identified. Like in yeast, these kinases can be activated by environmental stimuli such as UV-light, heat or osmotic shock. Currently three groups of MAP kinases are recognized, the classical MAP kinase ERK, stress-activated protein kinases (SAPK, or JNK) and p38. These molecules serve as transducers of signals from the cell membrane to the nucleus.

1.6.7.1   The ERK cascade

Extracellular signal-regulated protein kinases (ERK1, ERK2, also known as p44, p42 kinases) activate the transcription factor Elk-1 (Reimann et al., 1994). ERK is phosphorylated by its MAP kinase kinases, MEK1 and MEK2. The role of these kinases in the ERK cascade is illustrated by experiments with the MEK1,2 inhibitors, PD 098059 and Ro 09-2210. Blocking MEK1,2 results in inhibition of ERK activation (DeFranco et al., 1998; Van der Bruggen et al., 1999). MEK1 and MEK2 are activated by the serine/threonine kinase Raf-1. Raf-1 can be activated by tyrosine kinases (Reimann et al., 1994), Ras (Hall, 1994) or ceramide-activated protein kinase (CAPK) via ceramide (Kolesnick et al., 1994). In the process of MEK activation a small polypeptide called 14.3.3 ζ plays an important role (Luo et al., 1995).

It has been claimed that at least in the LPS-induced activation of the ERK cascade Raf-1 is not involved (Guthridge et al., 1997). In monocytes blocking studies demonstrated that inhibition of Raf-1 with genistein inhibited cytokine production. Complexes of Ras/Raf-1/MEK have been demonstrated during activation of ERK (Moodie et al., 1993) and transfection of a negative dominant mutant of Ras abolished LPS-induced TNF-α transcription (Frost et al., 1994). LPS-induced activation of monocytes was associated with a marked increase in the activity of Ras (Geng et al., 1994). Also in monocytes, LPS stimulation led to an increase in the tyrosine phosphorylation of the adapter protein Shc, which has been associated with the activation of Ras (Crowley et al., 1996). The ERK cascade is summarized in Diagram 1-15.
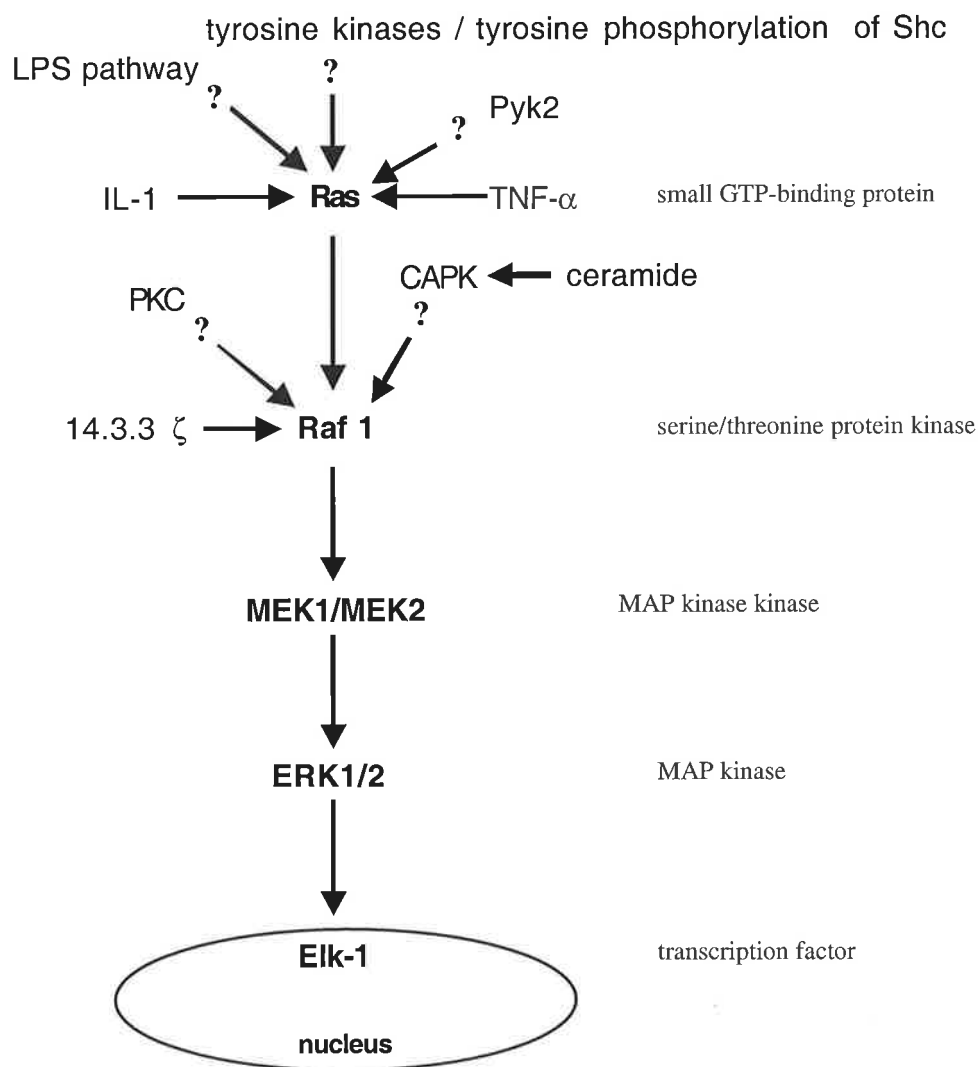
Diagram 1-15:   The ERK cascade. Signalling pathways are depicted as arrows. Unconfirmed pathways are denoted as " ? ". CAPK, ceramide-activated kinase; MEK, MAP-kinase ERK kinase; PKC, protein kinase C.

1.6.7.2 The JNK cascade

JNK, or c-jun NH_2-terminal kinase (also known as stress-activated protein kinase, SAPK), is another MAP kinase which activate transcription factors such as AP-1, consisting of c-jun, c-fos and ATF-2 (Kyriakis et al., 1994; Gupta et al., 1995; Adcock, 1997). The JNK cascade is activated by TNF-α, IL-1β and other proinflammatory mediators (Slowik et al., 1996). JNK itself is activated by MAP kinase kinases (MKK4, MKK7) (Sanchez et al., 1994; Holland et al., 1997), which themselves are activated by MAP kinase kinase kinases, such as MEKK1 and apoptosis-signalling kinase-1 (ASK-1) (Ichijo et al., 1997). Overexpression of MEKK1 has been reported to activate the NF-κB pathway (Read et al., 1997). As with the ERK cascade, the JNK cascade is activated by small GTP-bound proteins. Rac and Cdc42 activate the MAP kinase kinase kinases via several intermediate kinases of the PAK family (Diagram 1-16).
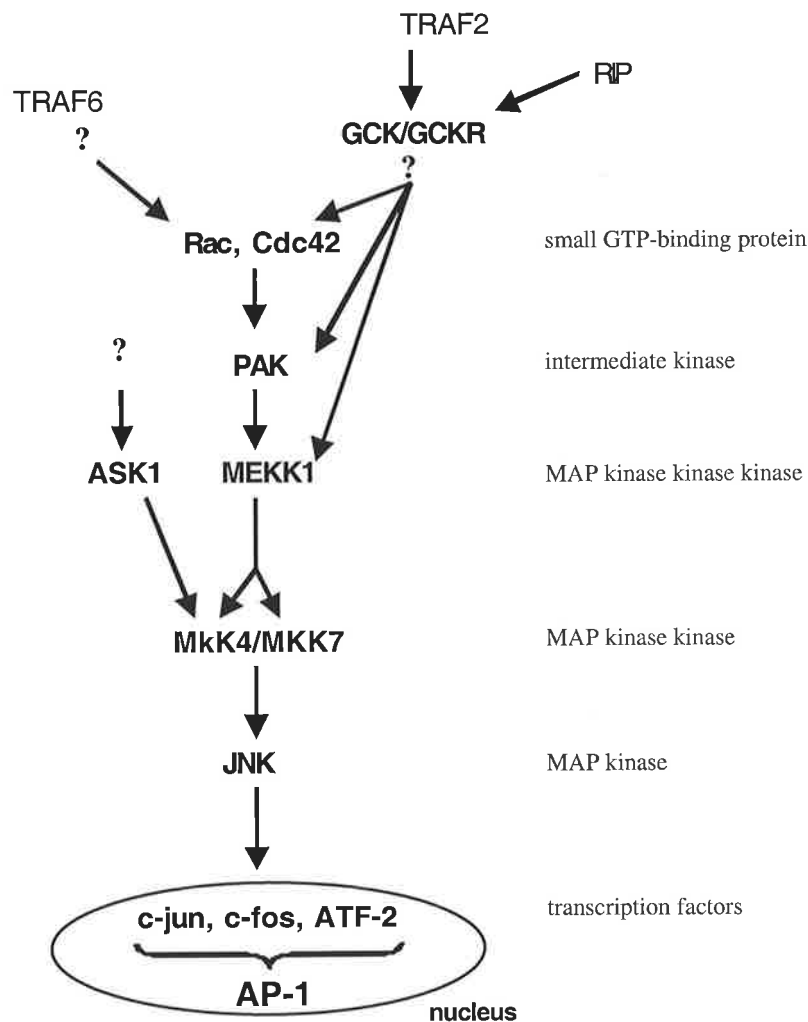
Diagram 1-16:   The JNK cascade. The small GTP-binding proteins Rac and Cdc42 activate MEKK1 and apoptosis-signalling kinase-1 (ASK1), probably via intermediate kinases of the PAK family. The MAP kinase kinase kinases activate MKK4 and MKK7, which phosphorylate JNK. JNK activates c-jun, c-fos and ATF-2, constituents of the transcription factor activator protein-1 (AP-1).

Overexpression of Rac or Cdc42, but not Ras, activates JNK (Min et al., 1997). Although TRAF2 is required for the activation of JNK (Natoli et al., 1997; Shi et al., 1997) it is not clear how receptor-associated factors such as RIP and the TRAFs connect with the JNK cascade. For TRAF2 direct activation of Rac and Cdc42 has been demonstrated (Pober, 1998). Recently other kinases downstream of TRAF2 has been described, germinal center kinase (GCK) and germinal center kinase-related (GCKR) (Pombo et al., 1995; Shi et al., 1997). When GCKR expression in cells was suppressed TNF-α- or TRAF2-mediated JNK activation was inhibited. It has been shown that TRAF2 directly interacts with the COOH-terminal regulatory domain of GCKR (Yuasa et al., 1998). While GCK or GCKR directly bind to MEKK1, their interactions with Rac, Cdc42 or PAK is not known (Kyriakis, 1999).

1.6.7.3   The p38 cascade

The MAP kinase p38, named after its molecular weight, was only cloned in recent years (Han et al., 1994). It predominantly activates transcription factor ATF-2 (Read et al., 1997). The MAP kinase kinases that activate p38 are SEK-1, MKK3 and MKK6. It is currently not known what the kinases upstream of these MAP kinase kinases are and how they are regulated (Diagram 1-17). TGF-β-activated kinase-1 (TAK-1) has been proposed as the main kinase (Yamaguchi et al., 1995; Kyriakis et al., 1996). ASK-1 has been demonstrated to phosphorylate MKK3 and MKK6 (Ichijo et al., 1997). Rac, CdC42 and PAK do not play a major role since inhibiting these proteins only resulted in a partial inhibition of p38 (Kyriakis et al., 1996). It is likely that there is some interaction between TAK-1 and the TRAFs and RIP since it has been shown that RIP alone can activate p38 and TRAF2-mediated activation of p38 requires RIP (Yuasa et al., 1998).

The finding that p38 activation by LPS in monocytes is inhibited by the MEK1,2 inhibitor Ro 09-2210 is intriguing (Van der Bruggen et al., 1999). MEK1,2 are not known to phosphorylate p38 and it is therefore possible that Ro 09-2210 may not be specific and may interfere with the activity of other kinases downstream of TRAF2 but upstream of p38, such as MKK3, MKK6 or MEKK1 (Diagram 1-17).

TRAF6
?

TRAF2, RIP
?

Rac, Cdc42

PAK

TAK-1, ASK1   MAP kinase kinase kinase

SEK-1, MKK3/MKK6   MAP kinase kinase

p38   MAP kinase

ATF-2   transcription factor

nucleus
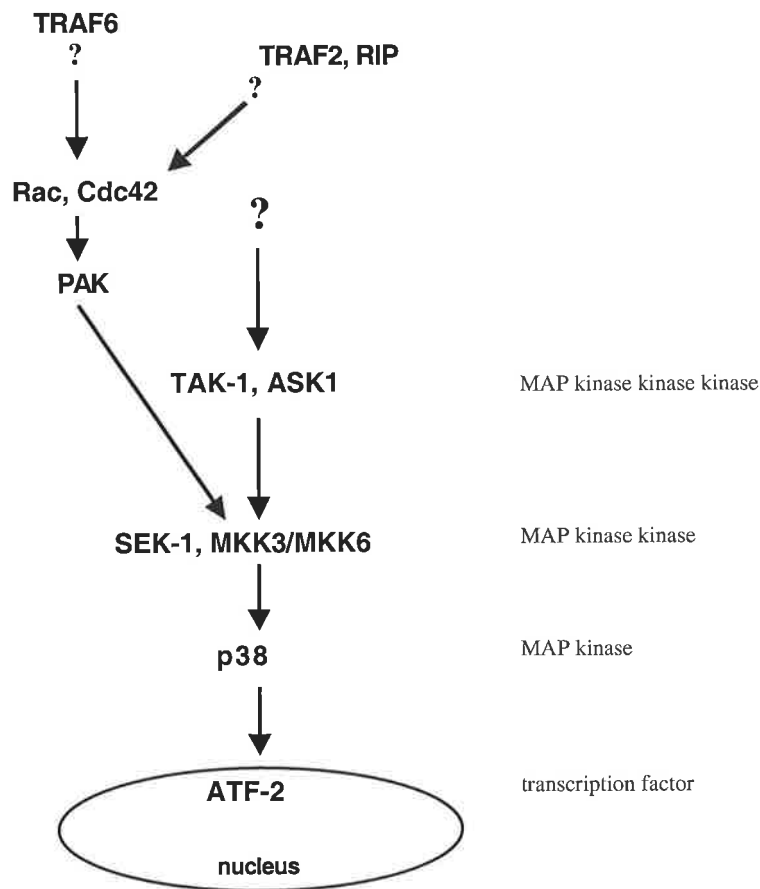
Diagram 1-17:   The p38 cascade. p38 is activated by the MAP kinase kinases MKK3 and MKK6. The main kinases upstream are TAK-1 (TGF-β-activated kinase1) and ASK-1, while Rac/CdC42/PAK only play a minor role. How TAK-1 and ASK-1 are activated is currently not known.

1.6.8   Special reference to signalling inindividual cell types

1.6.8.1 Monocytes and macrophages

Proinflammatory cytokines and LPS stimulate production of TNF-α, IL-1β and IL-6 in monocytes and macrophages. Expression of these cytokines is under the transcriptional control of NF-κB (Baeuerle et al., 1994; O'Connell et al., 1998). LPS-induced cytokine production has been studied in detail and apart from NF-κB, transcription factor AP-1 via the ERK and JNK/p38 cascades is involved (Geng et al., 1994; Geppert et al., 1994; Hambleton et al., 1995; Sweet et al., 1996; DeFranco et al., 1998; van der Bruggen et al., 1999).

1.6.8.2 Neutrophils

TNF-α and IL-1β activate NF-κB in neutrophils (Osborn et al., 1989). LPS-induced activation of neutrophils is mainly mediated through NF-κB and the p38 cascade, whereas the ERK and the JNK cascades do not seem to be involved (Nick et al., 1996). Within minutes after exposure to LPS Rac activity is increased (Dusi et al., 1996). In the absence of MKK6, the MAP kinase kinase MKK3 activated in neutrophils, phosphorylating p38α. (Nick et al., 1999).

Several pathways are implicated in the neutrophil respiratory burst and activation of the NADPH complex. These are PKC, ERK and p38 as well as tyrosine kinase-dependent activation of phospholipase A$_2$ (PLA$_2$) (Dusi et al., 1996). PLA$_2$ activation is also mediated by G-proteins and PKC (Osborn et al., 1989).

Although the mechanisms by which neutrophil priming by cytokines occurs have not been clearly defined there is some evidence that TNF-α and GM-CSF-induced activation of phospholipase D (PLD) and the subsequent increase in intracellular phosphatidic acid account for the enhanced response, at least to fMLP (Bourgoin et al., 1990; Bauldry et al., 1991).

1.6.8.3 Respiratory epithelial cells

In the cell line A549, TNF-α, IL-1β, IFN-γ and respiratory syncytial virus (RSV) upregulate ICAM-1 (Vorarberger et al., 1991; Chini et al., 1998). This is mainly mediated through activation of NF-κB, whereas unlike in endothelial cells the transcription factor AP-1 does not seem to play a role (Ray et al., 1993; Newton et al., 1995). Similar observations have been made with regards to the transcription of the IL-6, IL-8, NOS and urokinase genes (Asano et al., 1994; Guerrini et al., 1996; Newton et al., 1995). Recently it has been demonstrated in stimulation experiments with gram-negative bacteria (*P. aeruginosa*) that apart from NF-κB there is activation of Ras and MEK1/MEK2, but not JNK or p38. Blocking of this activation led to a 70% reduction in mucus production by respiratory epithelial cells (Li et al., 1998).

Respiratory epithelium is known to be relatively insensitive to LPS. Although LPS is able to upregulate ICAM-1 in hepatocytes, Kupffer cells and endothelial cells it has no effect on ICAM-1 expression in alveolar type II cells (Essani et al., 1995). LPS has been implicated in increasing the expression of inducible nitric oxide synthetase (iNOS) (Asano et al., 1994), increasing C3 by 100% (Rothman et al., 1989) and increasing epithelial IL-8 production five-fold (Palmberg et al., 1998). However, the signalling pathways involved have not been studied.

Unlike cytokines PMA-induced upregulation of ICAM-1 is dependent on PKCς. When levels of PKCς in A549 cells are depleted by antisense nucleotides PMA-induced upregulation of ICAM-1 is reduced (Dean et al., 1993). PMA increases the activity of transcription factor NF-κB (Newton et al., 1995).

1.6.8.4 *Vascular endothelial cells*

Endothelial cells express the IL-1 receptor, IFN-γ receptor and both TNF receptors. Interestingly, it has been demonstrated recently that they also express hTLR2 and hTLR4 (Zhang et al., 1999) and CD14 (Jersmann et al., 2001; see also Chapter 8).

It is currently believed that the upregulation of the endothelial adhesion molecules E-selectin, ICAM-1 and VCAM-1 in response to proinflammatory cytokines and LPS is controlled by NF-κB (predominantly p65) as well as JNK and p38 MAP kinase, which lead to the activation of transcription factor AP-1 (Collins et al., 1993; Reimold et al., 1996; Read et al., 1997). Experiments interfering with either NF-κB or JNK or p38 provide compelling evidence that each of the three pathways alone is required for maximal expression of E-selectin, ICAM-1 and VCAM-1. However, each signalling pathway is not sufficient for stimulating adhesion molecule expression (Whelan et al., 1991; Read et al., 1994; Chen et al., 1995; Min et al., 1997; Boyle et al., 1998; Tamura et al., 1998).

There has been some controversy whether ERK plays a role in endothelial cell activation. Although there are reports describing an increase in the activity of ERK after stimulation with TNF-α or LPS (Arditi et al., 1995; Schumann et al., 1996; May et al., 1998) inhibition of ERK does not interfere with endothelial adhesion molecule expression. Likewise, PKC does not play a major role in the upregulation of endothelial adhesion molecules (Montgomery et al., 1991; Ritchie et al., 1991).

1.6.9   Ceramide and sphingomyelinase

In recent years there has been a lot of interest in the role of the second messenger ceramide in cell signalling by cytokines and LPS. TNF-α and IL-1β cause an accumulation of ceramide through the action of sphingomyelinase on cell membrane sphingomyelin. This leads to an increase in activity of ceramide-activated protein kinase (CAPK) and PKC, implicated in activating Raf-1 and NIK (Lozano et al., 1994; Ballou et al., 1996; Modur et al., 1996). Exposure of cells to LPS leads to activation of CAPK without the generation of ceramide which led to speculations that LPS may enter cells and act by mimicking ceramide (Joseph et al., 1994; Kolesnick et al., 1994; Wright et al., 1995). However, treating cells with ceramide did not reproduce the effects of TNF-α and only incompletely and weakly mimicked LPS (Barber et al., 1996; MacKichan et al., 1999).

Interestingly, a new pathway linking ceramide to TNF-α-induced activation of cells has recently been discovered. Sphingosine 1-phosphate (S1P), generated from sphingosine via sphingosine kinase, mimics the effects of TNF-α when added to endothelial cells. In the reverse, blocking sphingosine kinase reduced TNF-α-induced upregulation of endothelial adhesion molecules by about 50% (Xia et al., 1998). In contrast, others have observed that IL-1-induced ceramide generation enhances but does

not trigger upregulation of E-selectin (Masamune et al., 1996). It is currently not known whether activation of cells by IL-1 or LPS leads to an increase in S1P.

1.7   THE ENDOTOXIN RECEPTOR CD14

1.7.1   Introduction

CD14 was first described as a myeloid differentiation antigen when it was first recognized to be expressed on pro-monocytes and thereafter on all monocytes and tissue macrophages including alveolar macrophages (Griffin et al. in 1981; Bernard et al., 1984; Ziegler-Heitbrock et al., 1993). CD14 is a 53-55kD glycoprotein with multiple leucine-rich repeats (leucine content 15.5%) and is expressed on many cell types (Setoguchi et al., 1989; Ferrero et al., 1990). The molecule consists of 356 amino acids and is encoded on chromosome 5 (q23-31) together with growth factors such as IL-3, GM-CSF and growth factor receptors such as the endothelial growth factor receptor, the β_2 adrenergic receptor and the platelet-derived growth factor receptor (Ferrero et al., 1988; Goyert et al., 1988). CD14 is attached to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor which is encoded on the X chromosome (Haziot et al., 1988; Takeda et al., 1993).

Deletion mutagenesis studies have demonstrated that the N-terminal 152 amino acids of CD14 are sufficient for mediating LPS-induced responses (Juan et al., 1995a). The region of the human CD14 molecule constituting the essential part of the binding site for LPS has been located between amino acids 39 and 44 (Stelter et al., 1997), whereas others found it to be between amino acids 57 and 64 (Juan et al., 1995b; McGinley et al., 1995). The corresponding binding site of CD14 on the LPS molecule is the polysaccharide core.

1.7.2   The physiological role of CD14

Recently a physiological role for CD14 in the recognition and phagocytosis of apoptotic cells by mononuclear phagocytes has been identified. The evidence for this is derived from experiments where an anti-CD14 monoclonal antibody significantly imparted the ability of macrophages to phagocytose apoptotic lymphocytes. In contrast, there was no interaction between macrophages and viable lymphocytes. Furthermore, the interaction of macrophages with necrotic lymphocytes was not influenced by the anti CD14 monoclonal antibody. It could also be established that blocking the binding site for apoptotic cells within the CD14 molecule could block binding to LPS and it was concluded that the binding site for LPS and apoptotic cells must be either identical or closely associated (Heidenreich et al., 1997).

Interestingly, the expression of CD14 seems to be associated with cell survival, at least in monocytes. These cells express the highest numbers of CD14 of all cell types, between 29,000 and

42,000 per cell (Vasselon et al., 1997; Van Voorhis et al., 1983). Shedding of CD14 by monocytes is associated with apoptosis and experimental enzymatic removal of CD14 with phosphatidylinositol-specific phospholipase C evokes apoptosis (Devitt et al., 1998).

Another surprising discovery implicates CD14 in the binding of IL-2 to human monocytes. Furthermore, blocking of CD14 with anti-CD14 antibodies prevents IL-2-induced monocyte activation. Also, transfection of CD14 into a IL-2-unresponsive human promonocytic cell line (U937) conferred responsiveness to IL-2 (Bosco et al., 1997). It has been proposed that IL-2 may be the physiological counterpart of LPS (Cox et al., 1990).

1.7.3   CD14 as a pattern recognition receptor for bacterial products

Membrane-bound CD14 (mCD14) has been identified as a receptor for lipopolysaccharide (Wright et al., 1990). The binding of FITC-labelled LPS can be competitively inhibited by 1000-fold excess unlabelled LPS, polymyxin B, bactericidal/permeability-inducing protein (BPI), cationic protein 18 and soluble CD14 (Troelstra et al., 1990). Apart from LPS, CD14 has been shown not only to bind to numerous other bacterial products, bacterial cell wall fragments and whole bacteria but also to mediate cell activation induced by these substances (Wright et al., 1990) (Table 1-11). The LPS-binding region within the molecule is remarkably preserved across species, with a high degree of gene sequence homology (Grünwald et al., 1993; Ikeda et al., 1997), suggesting it has a role in innate immunity, probably as a 'pattern recognition' receptor (Ulevitch et al., 1995).

Table 1-11: Other bacterial products and whole bacteria which are recognized by CD14

Bacterial product	Reference
Lipoarabinomannam	Savill et al., 1992
Muramyl peptide	Weidemann et al., 1997
Soluble peptidoglycans	Delude et al., 1995
Streptococcal wall polysaccharides	Savill et al., 1990
Lipoteichoic acid (LTA)	Nishikawa et al., 1990
Gram-positive cell walls components	Fadok et al., 1992

Bacterium	Reference
Mycobacterium. tuberculosis	Peterson et al., 1995
Salmonella. typhimurium	Onozuka et al., 1997
Helicobacter pylori	Bliss et al., 1998
Borrelia burgdorferi	Wooten et al., 1998
Bordetella. pertussis	Saadi et al., 1996

1.7.4 The role of CD14 in vascular disease

Recently two independent groups found an unexpected association between CD14 and cardiovascular disease. A polymorphism in the promoter of the CD14 gene was associated with myocardial infarction and furthermore asymptomatic people with that polymorphism had a significantly higher expression of CD14 on the surface of their peripheral blood monocytes (Unkelbach et al., 1999; Hubacek et al., 1999).

1.7.5 Soluble CD14

A form of CD14 without the GPI anchor (soluble CD14, sCD14, MW 48-50kD) is found in normal human serum at 2-6µg/ml, >90% originating from shedding of cell surface CD14 by monocytes and macrophages (Bazil et al., 1986; Schütt et al., 1992; Arditi et al., 1993). There is an increase of sCD14 found in adult respiratory distress syndrome (ARDS) and infections, and it has been observed that the levels of sCD14 in the serum of patients with gram-negative sepsis are associated with high mortality (Martin et al., 1994; Landmann et al., 1995).

1.7.6 Signalling and CD14

CD14 has been shown to mediate cell signals induced by LPS, such as tyrosine phosphorylation of proteins, activation of MAP kinases and activation of NF-κB in both stimulation and blocking experiments (Stefanova et al., 1991; Weinstein et al., 1992; Delude et al., 1995; Schumann et al., 1996). However, it is not clear through which mechanisms CD14 signals, since glycosylphosphatidylinositol anchors possess no transmembrane domain and are not regarded as capable of signalling (Wright, 1991) (Diagram 1-18).

The nature of the membrane anchor for CD14 is not relevant for LPS binding and LPS-induced cellular activation. This was demonstrated in transfection studies in murine pre-B cells (70Z/3) where the GPI anchor was replaced with either a tissue factor domain (CD14TF) or a class I domain (CD14CI) and all transfectants were rendered equally sensitive to LPS (Lee et al., 1993; Tobias et al., 1994). LPS-binding protein (LBP), a 60kD protein present in normal human serum at 10µg/ml, enhances the effect of LPS but is not required for LPS-induced cell activation, whereas CD14 is both sufficient and required (Tobias et al., 1992; Haziot et al., 1993).

Currently CD14 is thought to act as a lipid transfer protein, acquiring LPS from LBP and passing it onto a transmembrane signalling receptor (Hailman et al., 1994; Wurfel et al., 1997; Yu et al., 1997; Modlin et al., 1999).
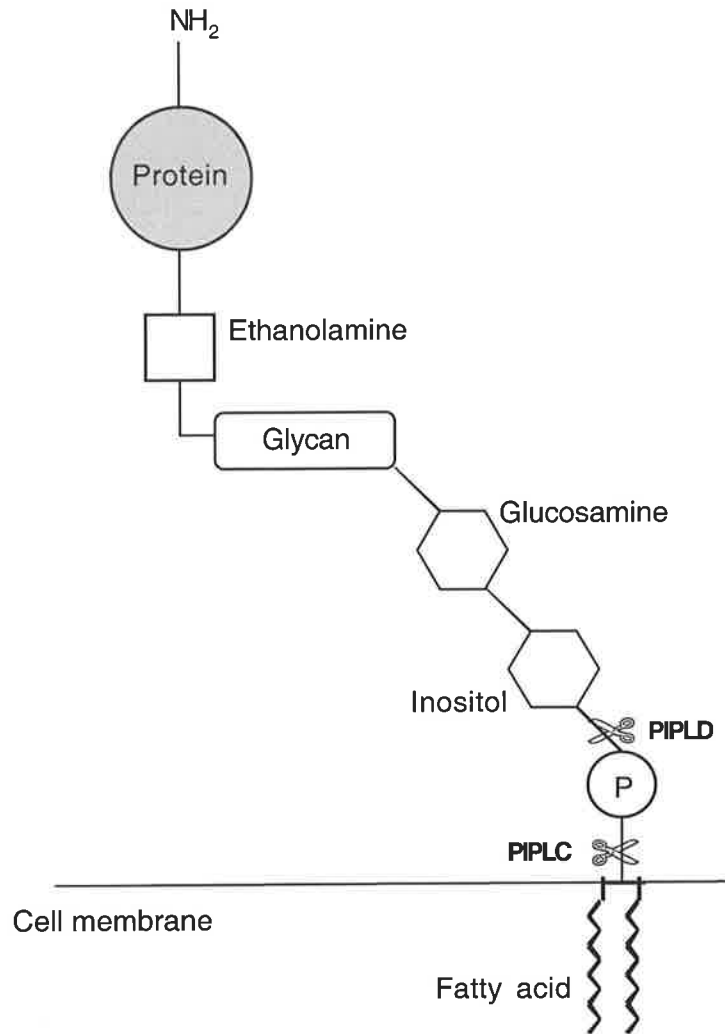
Diagram 1-18:   Composition of glycosylphosphatidylinositol (GPI) anchors. The protein component is variable, the anchor structure is highly conserved. P, phosphate; PIPLC, phosphatidylinositol-specific phospholipase C; PIPLD, phosphatidylinositol-specific phospholipase D; ✂, sites susceptible to enzymatic cleavage.

However, blockade of CD14 inhibits LPS-mediated effects nearly completely, suggesting that the signalling transmembrane receptor requires CD14, either as lipid transfer agent because it may have a low affinity for LPS or perhaps because the spatial relationship of the signalling receptor to CD14 may be important.

1.7.7   Signalling by other GPI anchored proteins

GPI-anchored proteins do not possess a transmembrane domain and are generally believed not to be capable of signalling, yet, there has been a growing body of evidence that many GPI-linked proteins other than CD14 are also implicated in signalling. A list of those molecules is compiled in Table 1-12.

Table 1-12: GPI-linked molecules other than CD14 implicated in cell signalling

Protein	Reference
Placental alkaline phosphatase (PLAP)	Harder et al., 1998
Influenza virus haemagglutinin (HA)	Harder et al., 1998
CD24	Fischer et al., 1990
CD48	Stefanova et al., 1993
CD55	Stefanova et al., 1993
CD59	Harder et al., 1999
CD73	Robinson et al., 1991
FcR III	Huizinga et al., 1988
LFA-3	Robinson et al., 1991

1.7.8   Is CD14 a raft protein ?

'Raft' is the term assigned to laterally assembled glycosphingolipids and cholesterol, which form micro-domains within the plasma membrane of a cell. Such structures possess a high lateral mobility in the fluid phospholipid bilayer and are insoluble with Triton X 100. Rafts typically contain GPI-linked proteins. Cross-linking experiments with those molecules have revealed coalescence of rafts to plaques accompanied by accumulation and activation of protein tyrosine kinases such as kinases of the Src family, consistent with the initiation of signalling cascades (Brown, 1993; Simons et al., 1997; Harder et al, 1998 and 1999). Thus the model of rafts has been proposed to explain the observed signalling associated with ligand-GPI-linked receptor interactions. To ascertain if CD14-mediated signalling utilizes rafts studies would be required whether cross-linking with CD14 antibodies mimics LPS-induced cell activation. In human monocytes anti-CD14 antibody rapidly induces a transient oxidative burst, consistent with the concept of micro-cluster formation (Schütt et al., 1988). It has also been reported that human bronchial epithelial cells (HBEC) produced IL-6 and IL-8 when exposed to sCD14 alone. However, it was not stated whether the sCD14 which was prepared from human urine was tested for impurities, such as LPS (Striz et al., 1998). Likewise human monocytes could be activated by sCD14, but again the LPS content of the sCD14 preparation was not stated (Landmann et al., 1995).

1.7.9   Could CD14 be a co-receptor for other membrane proteins ?

An alternative concept to cross-linking for CD14-mediated signalling would be the association of CD14 with a signal-capable trans-membrane protein. This interaction is facilitated by the high lateral mobility GPI-linked molecule display in the plasma membrane. An association of CD55 and CD59 with other membrane proteins has been demonstrated (Stefanova et al., 1991). Also, FcR III has been shown to associate with urokinase-type plasminogen-activated receptor (Xue et al., 1994). As far as

CD14 is concerned such association has only been documented with moesin using cross-linking and co-precipitation experiments (Tohme et al., 1999).

1.7.10   CD14 has a potential role in bacterial dissemination

When genetically CD14-deficient mice (CD14 -/-) were infected with gram-negative bacteria it was anticipated that these animals would undergo a less severe inflammatory response than CD14 expressing mice. In turn it was expected that the CD14-deficient animals would have a larger bacterial load. Surprisingly these animals showed reduced dissemination of bacteria compared to normal mice (Haziot et al., 1996). This observation is consistent with CD14 having a major role in dissemination of bacteria, perhaps through direct invasion of endothelial cells.

1.7.11   Soluble CD14 as a potential therapeutic agent in gram-negative infection

In human monocytes sCD14 inhibits LPS-induced chemiluminescence in a dose-dependent manner (Schütt et al., 1992; Grünwald et al., 1993). In animal studies sCD14 reduced the detrimental effects of experimental sepsis (Haziot et al., 1995).

1.7.12   *In vivo* trials with anti-CD14 antibody

When primates were pre-treated with anti-CD14 monoclonal antibodies prior to an endotoxin challenge the clinical symptoms and signs of shock, hypotension, altered pulmonary epithelial permeability and blood cytokine levels were prevented (Leturcq et al., 1996). Murine anti-CD14 protected mice from the effects of an endotoxin challenge (Matsuura et al. 1993). In a rabbit model of endotoxin shock produced by multiple exposures to endotoxin anti-CD14 monoclonal antibodies protect against organ injury or death. This protection was achieved even when the antibody was administered after exposure to LPS. In contrast, anti-TNF-α antibody conveyed no protection in these rabbits (Schimke et al., 1998).

1.8   THE NORMAL LUNG AND THE LUNG IN INFLAMMATION.

1.8.1   Functions of the lung

The lungs main function is gas exchange. However, it also has an important role in host defense being one of the three major interfaces between the host and the environment, together with the dermis and the gastro-intestinal tract. From the clinical perspective the lung appears to be a relatively simple organ, which can only respond in limited ways to a myriad of disease processes. Its main responses are

airway narrowing (mucus production, bronchoconstriction), interstitial thickening (acute oedema, chronic scarring), loss of parenchyma (emphysema) and occupation of alveolar airspace with material other than air (pneumonia, alveolar proteinosis). A closer look, however, reveals the lung to be an organ with fascinating complexity. This becomes apparent if one looks only at two aspects, the structural elements constituting the organ or the main functions the lung has for the entire organism (Tables 1-13 and 1-14).

Table 1-13:   Cell systems forming the structure of the lung

Structural element	Cell type	
Epithelium	Clara cells Goblet cells Type I and Type II pneumocytes Neuroendocrine cells, Myo-epithelial cells)	
Smooth Muscle Cells	Bronchial smooth muscle cells Vascular smooth muscle cells	
Fibroblasts	Connective tissue cell, lung matrix	
Endothelium	Microvascular, capillaries Pulmonary artery	
Neurons	Non-adrenergic/non-cholinergic system, [NANC]	
Extracellular Matrix	Basement membrane	
Immune Cells	Resident	Bronchus-associated lymphoid tissue [BALT], tissue macrophage, mast cells, alveolar macrophage
	Migratory	neutrophils, macrophages, lymphocytes, eosinophils

Table 1-14:   Main functions of the lung

Task	Function and effect
Gas Exchange	Take-up of O_2, exhaust CO_2
Surfactant Production	Reduced surface tension, anti-micobial activity
Immunity	Defense against inhaled pathogens, antigen presentation
Barrier	Muco-ciliary inactivation and clearance
Endocrine	Angiotensin II, serotonin, substance P production
Coagulation	Tissue factor production

1.8.2   The lung and infection

Pathogens are constantly present in air, either as spores or droplets. The mucosal immune defense consists of mucus with soluble mediators such as surfactant, IgA and enzymes such as small proteases like Elafin. The epithelial cells form a barrier facilitating ciliary clearance and pathogen removal by alveolar macrophages and tissue macrophages.

1.8.3   Pneumonia

1.8.3.1 Introduction

Most potential pathogens, which are inhaled, do not cause disease. Depending on the immune system of the host and/or the ability of the inhaled bacterium to adhere to the airway epithelial cells and escape destruction, infection rapidly spreads from the initial focus. Either an entire lobe of lung is homogenously involved (lobar pneumonia), or the infection has multiple foci around terminal bronchi (bronchopneumonia). The infection leads to temporary solidification of the involved segment of lung (red hepatization, gray hepatization). In most cases resolution of the process begins between 5 and 10 days, often leading to recovery without sequelae after 3-4 weeks. However, occasionally the affected lung can undergo organization, leading to permanent scars, sometimes to bronchiectasis. Depending on the circumstances the mortality of pneumonias caused by gram-positive organisms ranges from 15-40% and from 50% or higher for gram-negative bacteria.

1.8.3.2 The immune system in lung infection

When pathogenic microorganisms enter the airway most will be inactivated and destroyed by mucosal IgA, surfactant and other bactericidal proteins. However, some organisms may make contact with respiratory epithelial cells and alveolar macrophages. These cells secrete pro-inflammatory cytokines such as TNF-α, IL-6, IFN-γ and IL-1β in response to microbial products (Sibille et al., 1990). These cytokines cause activation of other local tissue cells as well as resident immune cells, which then produce more cytokines and also chemoattractants, like IL-6 and IL-8. This leads to the rapid recruitment of more inflammatory leukocytes, predominantly neutrophils, to the focus of inflammation (Sharma et al., 1992). This process is aided by the upregulation of cellular adhesion molecules on both, vascular endothelial cells and leukocytes. In addition, TNF-α causes an increase in permeability of pulmonary epithelium (Li et al., 1995).

Although the armory of leukocytes is designed to inactivate bacteria, proteases, nitrogen derived intermediaries and oxygen radicals released in large quantities also damage local tissues and leukocytes. This causes an accumulation of secretions, proteins and pus, conditions favouring

proliferation of microorganisms. Such area of lung must be walled-off, the secretions and necrotic material removed through expectoration. The droplets generated by coughing may then be inhaled by other individuals, hence propagating the causative organism. After several hours other cell types, such as monocytes and lymphocytes migrate to the infective site in the lung. Systemic effects of the local process are stimulation of hepatocytes by IL-6, TNF-α and IL-1β to produce acute-phase proteins. Systemic reactions like fever are caused by the resetting of thermostat-like neurons in the hypothalamus by pyrogens such as IL-1.

Finally, after about three weeks the pulmonary infection clears with little or no residual organ damage. Given the size of the neutrophil infiltrate in pneumonia this is remarkable and is achieved by removal of neutrophils by apoptosis (Grigg et al., 1991; Cox et al., 1995).

Apart from acute infections with an inflammatory response to LPS there is evidence that chronic exposure to LPS (like in organic dusts) may lead to pulmonary hypertension or chronic lung diseases such as emphysema (Meyrick et al., 1986; Milton et al., 1990; Jagielo et al., 1998).

1.8.3.3 Gram-negative sepsis

Successful mucosal invasion causes bacteriaemia, often leading to the clinical syndrome of sepsis. Sepsis is defined as an infection-induced syndrome with two or more of the following features being present: fever or hypothermia, leukocytosis or leukopenia, tachycardia and tachypnoea or an increased minute ventilation (Wheeler et al., 1999).

In infections with gram-negative bacteria the main mediator of shock is LPS. Although LPS can activate cells per se, its main action during sepsis is thought to be mediated through TNF-α (Beutler et al., 1985). TNF-α serum levels correlate with fatal outcomes (Waage et al., 1987). In as many as 40% of cases of gram-negative sepsis the anti-inflammatory response mounted fails to clear the infection and circulatory shock, multiorgan failure and death occur (Marsh et al., 1996). Even when the origin of bacterial dissemination is situated elsewhere, the lung is usually the first organ to fail (Welbourn et al., 1992). Nearly 85% of patients with gram-negative sepsis require ventilatory support and almost half develop ARDS (Wheeler et al., 1999).

1.8.4   Overview of current approaches to the treatment of gram-negative infection/sepsis

1.8.4.1 Conventional therapies

The mainstay of treatment for sepsis in western medicine are antibiotics. After obtaining blood cultures in order to later base treatment on the sensitivity of the identified organism, empirical treatment is started immediately. Generally a broad spectrum penicillin or cephalosporin in combination with gentamicin are administered intravenously. The role of corticosteroids and positive

inotropes remains contoversial. According to current evidence they have little or no role in the treatment of sepsis or other serious systemic bacterial infections.

There is a worldwide increase in bacterial resistance to antibiotics. While the reasons for this phenomenon are speculative and range from blaming over-prescribing by medical practitioners to blaming commercial use of antibiotics in animal farming there is a growing need for alternative means to control bacterial infections (Kingman, 1994; Stone, 1994; Nuorti et al., 1998). The fact, that there are more immune-compromised patients than ever (the elderly, patients receiving chemotherapy for malignant diseases, organ transplant recipients, patients with HIV infection) compounds the issue. Therefore, the development of new therapies to control bacteria is one of the greatest challenges facing modern medicine today.

1.8.4.2 Novel therapies that target inflammatory mediators

Many new, specific therapies are based on research into the pathophysiology of bacterial infection. One approach focuses on TNF-α. TNF-α itself proved too toxic at doses required to stimulate the immune system. Trials with monoclonal antibodies against TNF-α and soluble TNF receptors were tried in the past decade but were disappointing. Currently a TNF-α mimetic (Peptide 419) is under clinical evaluation and achieved good preliminary results in a murine model of *Pseudomonas aeruginosa* pneumonia (Ferrante and Rathjen, unpublished data).

Another avenue of treatment options focuses on LPS. Both, anti-LPS or anti-lipid A monoclonal antibodies and non-toxic LPS or lipid A molecules have been tried with moderate success.

A third approach is to administer soluble CD14 (sCD14), or, more recently, antiCD14 monoclonal antibody. The latter produced impressive success in mouse, rabbit and baboon models. Other options include PAF antagonists, IL-1 receptor antagonists, GM-CSF as non-specific augmentation of the immune response, and human recombinant BPI. A synopsis of the various biological therapies for gram-negative infection and sepsis is provided in Table 1-15.

Table 1-15:   Experimental treatments for gram-negative bacterial infection and sepsis

Treatment	Reference	Outcome
Strategies based on antagonizing TNF		
anti-TNF mab	Siegel et al., 1995; Fekade et al., 1996	+
soluble TNF receptors	Mohler et a., 1993; Opal et al., 1996	= (+)
TNF receptor:FC fusion protein	Fischer et al., 1996; Abraham et al., 1997	=
TNF-mimetic peptide 419	unpublished data	+
Thalidomide	Moreira et al., 1997	+

thalidomide analogues	Corral et al., 1996	+
Pentoxifylline	Staubach et al., 1998	+

Strategies based on antagonizing LPS

LPS antagonist E5531	Christ et al., 1995; Kawata et al., 1995	+
anti-LPS mab HA-1A	Ziegler et al., 1991	= (+)
anti-LPS mab E5	Greenman et al., 1991	= (+)
anti-LPS mab T88	Glauser, 1996	=
anti LPS mabs D6B3, D6B4 anti lipid A mab lipid A analogue SDZ MRL 953	Vacheron et al. 1992 Teng et al., 1985 Stern et al., 1995	+ (+) +
endotoxin neutralizing proteins (ENP), recombinant *Limulus polyphemus* anti-LPS factor (LALF)	Nelson et al., 1995; Weiner et al., 1996	+
Polymyxin B (PmB)	Baldwin et al.,1991	+ (toxic)
synthetic PmB-peptide	Rustici et al., 1993	+
extra-corporal LPS-adsorption	Pollack et al., 1996	(+)
lipoproteins (HDL)	Quezado et al., 1995	= ↓

Strategies based on serum LPS binding proteins

anti-LBP mab human recombinant BPI	Gallay et al., 1993 Dahlberg et al., 1996; Giroir et al., 1997	+ (if given at onset) +
Cationic antimicrobial protein 18	Tasaka et al., 1996	(+)

CD14-based therapies

soluble CD14	Goyert et al., 1995; Haziot et al., 1995	+
anti-CD14 mab	Matsuura et al., 1993; Leturcq et al., 1996; Schimke et al., 1998	++

Strategies based on cytokines other than TNF

PAF receptor antagonist	Dhainaut et al., 1994	(+)
GM-CSF	Nelson et al., 1998	(+) with AB
IL-1 receptor antagonist	Ohlson et al., 1990; Fischer et al., 1994;	+

Opal et al., 1996

Miscellaneous approaches

non-steroidal anti-inflammatory drugs (NSAIDS) (Ibuprofen)	Bernard et el., 1997	=
Vaccinia virus-mediated overexpression of TNF-α and IFN-γ	Cheers et al., 1999	+
Antioxidants (N-acetylcysteine)	Vincent, 1997	=
Tyrosine kinase inhibitor	Novogrodsky et al., 1994	+
Plasmapheresis	Pollack et al., 1996	

(Legend: = ↓ no change or even worse outcome;   = no change;   = (+) minimal benefit;
       (+) slight benefit;   + beneficial;   ++ striking benefit.)

1.9   HYPOTHESIS, AIMS AND SIGNIFICANCE

Regulation of inflammatory responses by exogenous and endogenous mediators has previously concentrated on the effects of the action of a single mediator on cellular responses (Pober et al., 1990; Schulze-Osthoff et al., 1997, Kyriakis, 1996). This represents a limitation in the understanding how inflammatory responses are regulated. LPS is a strong inducer of TNF-α and hence these two cell stimulators are present concomitantly in fluids and tissues during infection (Richter et al., 1989; Krüll et al., 1996; Blank et al., 1997). Even in non-infectious situations, such as trauma with circulatory shock, significant amounts of bacterial products such as LPS translocate from the intestinal compartment (Bahrami et al., 1995; Martin et al., 1997). The focus of the work presented in this thesis was directed towards the evaluation of the effects of concomitant exposure of cells relevant to lung inflammation to LPS and TNF-α.

1.9.1   Hypothesis

It is evident from previous findings that both TNF-α and LPS have the ability to alter cellular responses to other agonists, usually these responses are increased. It is therefore feasible that TNF-α and LPS also increase responses to each other. We thus postulate that (i) TNF-α and LPS act synergistically to stimulate cytokine production, respiratory burst and adhesive properties of phagocytic cells. (ii) In the induction of adhesion molecules on endothelial and epithelial cells TNF-α

and LPS are synergistic. (iii) The mechanism of synergism is a function of increased surface expression of CD14 and/or the amplification of intracellular signaling pathways.

1.9.2   Specific aims

To test these hypotheses we will address the following aims:

(i)   To examine whether or not TNF-α and LPS act synergistically to stimulate neutrophil superoxide production and adhesion.

(ii)   To examine the effects of concomitant exposure of monocytes and macrophages to TNF-α and LPS on the production of TNF-α, IL-1β and IL-6.

(iii) To study the combined effects of TNF-α and LPS on the expression of E-selectin, ICAM-1 and VCAM-1 on human umbilical vein endothelial cells, and ICAM-1on a type II respiratory epithelial cell line.

(iv)   To investigate whether or not TNF-α modulates the expression of the LPS receptor, CD14, on neutrophils, monocytes, endothelial cells and epithelial cells.

(v)   To examine whether cells exposed to both TNF-α and LPS display unique differences in intracellular signals stimulated.

1.9.3   Significance

The results generated in this study are likely to lead to a much better appreciation of the mediator network operating in infection-induced inflammation with the potential to develop novel therapeutic strategies in the management of gram-negative infections, including pneumonia and sepsis.

Chapter 2

Materials and Methods

2.1   MATERIALS

2.1.1   Lipopolysaccharide and cytokines

Lipopolysaccharide from *Escherichia coli*, serotypes K-235, 055:B5 and 0127:B8, chromatographically purified by gel filtration, were purchased from Sigma Chemical Co., St. Louis, Mo. For LPS binding studies FITC-conjugated LPS (f-LPS) from E. coli 0127:B8, also from Sigma, was used. Lyophilized LPS powder and LPS stock solution (10mg/ml) were stored at -20^0C. The LPS working solution was kept at 4^0C.

Human recombinant TNF-α was a gift from Dr G R Adolf, Ernst Boehringer Institute, Vienna, Austria.   The activity was 6 x 10^7U/mg and the preparation was >99% pure.   1U of TNF-α is equivalent to 0.01ng or 3.48×10^8 molecules. The endotoxin contamination of the TNF-α preparation was less than 0.125 endotoxin units/ml as assessed by the limulus lysate assay. Recombinant human IL-1β was obtained from DuPont, De Nemours, France. Interferon-γ (IFN-γ) was obtained from Boehringer Mannheim, Germany. GM-CSF was purchased from Sandoz, Basel, Switzerland, 100ng contained 10^5 Units. All cytokines were stored at -70^0C.

2.1.2   Antibodies

The antibodies used in this thesis are listed in table form for easy reference (Table 2-1).

Table 2-1: List of antibodies

Specificity	Species	Label	Source
Von Willebrand Factor	rabbit anti-human IgG	HRP	DAKO, Denmark
E-selectin	mouse anti-human (mab)	pure	Becton Dickinson (BD), San Jose, CA
ICAM-1	mouse anti-human (mab)	pure	BD
VCAM-1	mouse anti-human (mab)	pure	BD
CD14 (MY4)	mouse anti-human (mab)	Pure and biotinylated	Coulter Corporation, USA
CD14 (TÜK4)	mouse anti-human (mab)	Pure or PE-conjugated	DAKO, Denmark
CD14 (2D-15C)	mouse anti-human (mab)	pure	Silenus-AMRAD Biotech, Melbourne, Australia
CD14 (Leu™-M3)	mouse anti-human (mab)	pure	BD

CD14 (RMo52)	mouse anti-human (mab)	pure	Immunotech, Marseille, France
Mouse IgG	Rat anti-mouse (mab)	biotinylated	BD
IgG$_1$ (isotype control)	mouse anti-human (mab)	pure	DAKO
IgG$_{2a}$ (isotype control)	mouse anti-human (mab)	Pure	BD
IgG$_{2b}$ (isotype control)	mouse anti-human (mab)	Pure	BD
IgG$_{2b}$ (isotype control)	mouse anti-human (mab)	PE-conjugated	DAKO
Mouse IgG	rabbit anti-mouse (affinity isolated)	HRP-conjugated	DAKO
Mouse IgG	sheep anti-mouse (affinity isolated)	FITC-conjugated	Silenus
Mouse IgG	rabbit anti-mouse (affinity isolated)	R-PE-conjugated	DAKO
NF-κB (p65-Rel)	rabbit anti-human (mab) (polyclonal)	pure	Santa Cruz Biotechnology, USA.
Rabbit IgG	sheep-anti-rabbit	HRP-conj.	Silenus
ERK	goat anti-human (polyclonal)	pure	Santa Cruz
p38	goat anti-human (polyclonal)	pure	Santa Cruz
CD11a	mouse anti-human (mab)	FITC-conj.	BD
CD11b	mouse anti-human (mab)	PE-conjugated	BD
CD11c	mouse anti-human (mab)	PE-conjugated	BD
CD18	mouse anti-human (mab)	FITC-conj.	BD
CD33	mouse anti-human (mab)	PE-conjugated	BD
CD45	mouse anti-human (mab)	FITC-conj.	BD

2.1.3   Other reagents

- 7-aminoactinomycin D (7-AAD), benzamidine, cycloheximide (CHX), Cytochrome C (type III, horse heart), DTT (DL-Dithiothreitol, Cleland's reagent), EDTA, endothelial growth supplement (ECGS), Folin's Solution (Ciocalteu's Phenol reagent, 2.0N), Leupeptin ([Acetyl-Leu-Leu-Arg-al] Trifluoroacetate salt), Lucigenin (bis-N-Methylacridinium Nitrate), lysophosphatidic acid (LPA), Nonidet P-40, pepstatin A, Phenylmethylsulfonyl fluoride (PMSF), PMA, Protein A sepharose beads, Sigma 104 (phosphatase substrate), superoxide dismutase (SOD), were all purchased from Sigma.

- Human serum albumin (HSA, Albumex®, 200mg/ml), human immunoglobulin G for non-specific blocking (Intragam™, 60mg/ml) and Bovine serum albumin (BSA), were obtained from Commonwealth Serum Laboratories (CSL), Parkville, Victoria, Australia.
- Acrylamide (N,N'-Methylbisacrylamid 30%), Ammonium persulfate (APS), sodium dodecyl sulfate (Natrium lauryl-sulfat, SDS) TEMED (N, N, N', N',-Tetramethylethylenediamine: Bio-Rad Laboratories, Hercules, CA, USA.
- Collagenase type II, 0.4mg/ml, activity 219U/mg, (dry enzyme was weighed out, dissolved in HBSS buffer and filter-sterilized [0.2µm]): Worthington, Freehold, NJ.
- Gelatin (sterile, 0.2%): TRACE Biosciences, Australia.
- Glutaraldehyde 2.5%: Probing and Stucture, Qld, Australia.
- Trypsin (porcine): ICN Flow, Aurora, O.
- Triton™ X100 and Tween® 20 (Polyoxyethylene(20) sorbitan monolaurate: Ajax Chemicals, Auburn, NSW, Australia.
- Nitrocellulose paper (Optitran BA-S83, reinforced NC): Schleicher & Schuell, Dassel, GER.
- ISOTON II solution: Coulter Electronics, Brookvale, NSW, Australia.
- Scintillation fluid: Fisher Chemicals, Loughborough, England
- ABTS buffer (2,2'-azino-di-[3 ethylbenzthiazoline sulfonate-(6)] and 0.012% hydrogen peroxide in citrate-phosphate buffer, pH 4.2): Boehringer Mannheim, Germany.
- The human E-selectin c-DNA probe was a 3.8-kb insert cloned into the *Xho-1* site of a pB-SK vector, was a gift from Dr J Gamble, Hanson Centre for Cancer Research, Institute for Veterinary and Medical Science, Adelaide.
- FITC Quantum™ 26 beads: Flow Cytometry Standards Corporation, San Juan, PR.
- Streptavidin-PE: Becton Dickinson.
- Glutathione-Sepharose 4B: Pharmacia Biotech.
- Enhanced chemiluminescence reagent: New England Life Science Products, Boston , Ma.
- GAPDH probe cocktail: Clontech.
- RNA$_{zol}$ B: Cinna-Biotrex.
- Gene Screen Plus nylon membrane and slot-blot vacuum manifold: Hoeffer.
- Denatured Herring sperm DNA: NEN, Du Pont, De Nemours, France.
- Lowry's Solution (2% Na_2CO_3, 1% SDS, 0.4% NaOH, 0.16% Na/K-tartrate).
- [α-^{32}P]dCTP and ^{35}S L-methionine: Amersham, Little Chalford, Buckinghamshire, UK.
- γ^{32}P-Adenosine 5'-Triphosphate (ATP): GeneWorks, Adelaide, South Australia, Australia.

2.1.4 Culture dishes

The following flasks and dishes were all sterile and of tissue culture grade:

- 75cm^2 culture flasks (Corning, Cambridge, Ma)
- 10cm tissue culture dishes (54 cm^2), Corning, USA.
- 3.5cm tissue culture dishes (9.62cm^2), Nunc..
- 6 well plates (9.62cm^2/well), Linbro-Flow, Aurora, O.
- 24 well plates (2cm^2/well), Linbro.
- 96 well plates (0.38cm^2/well), Linbro.
- Microtiter plates, Cooke, Dynatech Laboratories, Chantilly, Va.
- Chamber slides, Lab-Tek, Nunc, Naperville, IL.

2.1.5   Serum, culture media and buffers

Human group AB serum was a donation from Mr. Ian Bates of the Red Cross of South Australia. The serum from several donors was pooled, heat-inactivated for 30min at 56^{0}C, and stored in 10ml aliqots at -20^0C until used as culture media supplement for HUVEC. Fetal calf serum (FCS) was purchased from TRACE, Australia.

Hanks Balanced Salt Solution (HBSS), Phosphate Buffered Saline (PBS) and RPMI 1640 were all from Cell Image™, Adelaide, Australia, and were all endotoxin-free as assessed by the Limulus Amoebocyte Lysate assay. All cell culture media were supplemented with penicillin and streptomycin (80µ/ml) and L-glutamine (3.2mmol/L), all from ICN Pharmaceuticals, Costa Mesa, CA. All buffers and media were filtered through 0.2µm filters (Sartorius, Göttingen, Germany).

2.2   METHODS

2.2.1   Preparation of mononuclear leukocytes and neutrophils

Neutrophils were prepared by the rapid single step method (Ferrante et al., 1978). Heparinized blood from healthy volunteers (6ml) was layered over a solution of hypaque-ficoll (4ml, d = 1.114) and centrifuged at 400g/35min at room temperature. After centrifugation mononuclear cells were located in the first leukocyte band and the neutrophils in the second (Photograph 2-1). The cells were harvested, washed and resuspended in HBSS prior to use in experiments. The neutrophil preparations were >96% pure and >99% viable as judged by the ability to exclude trypan blue. The mononuclear fraction contained between 8% and 16% monocytes.

2.2.2   Purification of peripheral blood monocytes by adherence to plastic

Tissue culture dishes were coated with autologous plasma (37^{0}C, 30min). After discarding the plasma the cells contained in the mononuclear band of the leukocyte preparation were resuspended in RPMI

1640 medium (supplemented with 20% FCS) and transferred into the coated dishes. After 30min incubation lymphocytes were removed by washing the cell monolayers and the adherent monocytes were replenished with HBSS containing 2% autologous donor serum for cytokine producttion experiments.
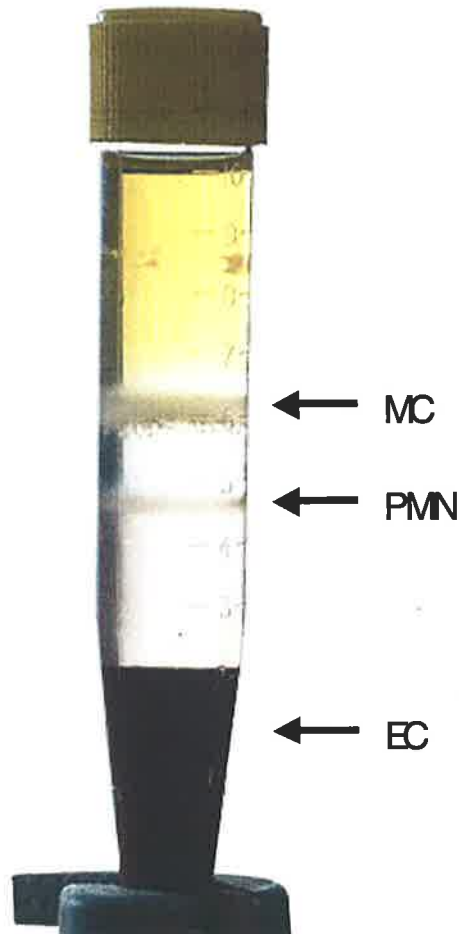
Photograph 2-1: Leukocyte bands obtained with the single step method. After 6ml of heparinized peripheral blood were layered onto 4ml of Hypaque-Ficoll gradient the tubes were centrifuged (35min, 400g). The supernatant is the plasma. PMN, neutrophil band; MC, mononuclear cells; EC, red cell pellet

2.2.3   Differentiation of monocytes into macrophages

After the purification of monocytes as described above the cells were kept in culture medium (RPMI 1640, 10% FCS, L-glutamine, P/S) for 4-6 days to allow differentiation of the monocytes into macrophages. If required to be transferred the cells were removed from the original Petri dishes with a cell scraper, counted, and seeded into appropriate tissue culture dishes for the indicated treatments.

2.2.4   Culture of Human Umbilical Vein Endothelial Cells (HUVEC)

It is inherently difficult to obtain primary cultures of human endothelial cells in sufficient quantities. Although parts of saphenous vein, which occasionally remain unutilized in bypass grafting, or vessels from amputed limbs have been used, these types of specimen are not widely available. In the search for a model suitable for *in vitro* studies of the endothelium a method of harvesting human umbilical cord veins endothelial cells (HUVEC) by enzyme digestion was introduced (Jaffe et al., 1973). Since then HUVEC have been widely adopted as a useful and valid experimental model.

The characteristics of the responses of HUVEC in terms of surface expression of adhesion molecules induced by proinflammatory mediators do not differ from those of cultured endothelial cells from human saphenous vein and human femoral artery, thus confirming HUVEC to be a relevant tool in endothelial research (Klein et al., 1994).

Human umbilical cords were collected immediately after delivery and stored in sterile containers at 4^0C for a maximum period of 36h. The veins were cannulated, washed with HBSS and filled with collagenase (37^0C, Collagenase type II, 0.4mg/ml, activity 219U/mg). After incubation in a water bath (37^0C, 2min) the content of each vein was collected (Photograph 2-2).

Photograph 2-2:   Preparation of HUVEC. Umbilical cords were cannulated and washed with buffer (HBSS, 37^0C). Then the cords were filled with collagenase, incubated for 2min and the enzyme/endothelial cell solution was collected.

The veins were washed once with HBSS to harvest any remaining cells. Cells from each cord were centrifuged separately (400g, 5min), the supernatant discarded and the pellet resuspended in RPMI 1640 (supplemented with 80U/ml penicillin, 80µg/ml streptomycin, 3.2mmol/L L-glutamine and 20%

pooled, heat inactivated human group AB serum. Cells were transferred into 75cm^2 culture flasks, which were pre-coated with 0.2% gelatin and grown to confluence (Photograph 2-3).
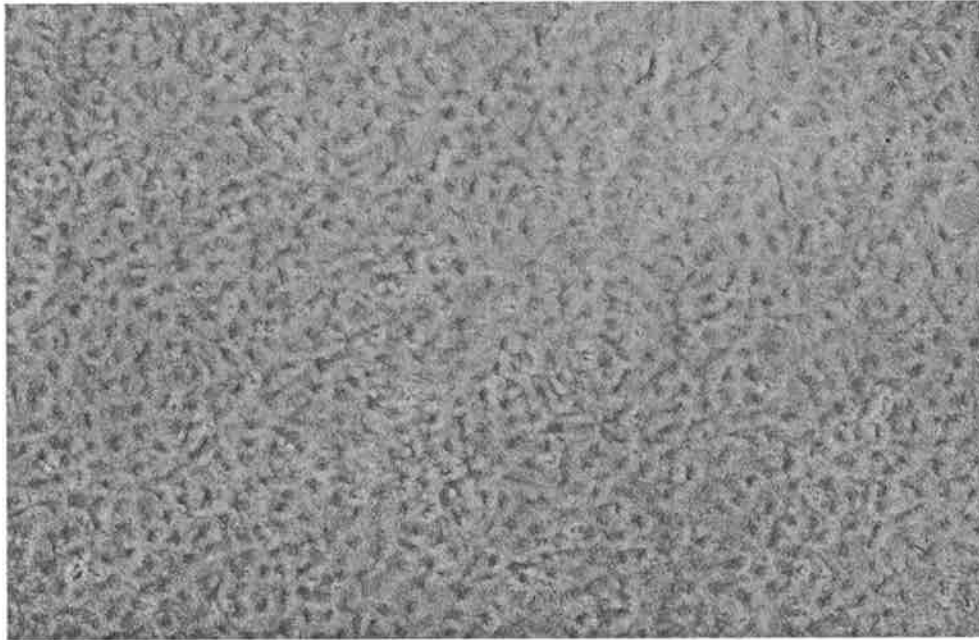
Photograph 2-3: Confluent HUVEC in culture. These cell were prepared 4 days earlier and have not reached the typical cobble-stone pattern yet. The photo was taken from a phase-contrast microscope (Optiplan, Leitz, Wetzlar), magnification 40x.

Endothelial cells were identified by their characteristic monolayer cobble stone appearance and positive staining for factor VIII-related antigen using peroxidase-conjugated rabbit IgG antibody to human von Willebrand factor and 3.3'-diaminobenzidine. Only first passage cells from one cord were used for one experiment. Prior to experiments, cells were harvested from culture flasks with trypsin (0.05mg/ml) and EDTA (0.02mg/ml).

2.2.5   The human endothelial cell line HUV-EC-C

The cell line HUV-EC-C (ATCC No CRL-1730) is derived from human umbilical vein endothelial cells and has a life expectancy of 50-60 passages. It is propagated in RPMI 1640 media supplemented with 10% FCS, Heparin (2.5%), ECGS (150µg/ml), penicillin (80U/ml), streptomycin (80µg/ml) and L-glutamine (3.2mmol/L)

2.2.6   Culture of the epithelial cell line A549 (ATCC CCL-185)

A549 cells are grown in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum, Penicillin/Streptomycin and Glutamine at 37^{0}C in 5% CO$_2$ humidified atmosphere. Coating of plastic

culture dishes is not necessary. Cells seeded at a density of 1:4 become confluent in 2-4 days. Prior to subculturing the cells were washed in warm HBSS to remove medium and debris. Trypsin/EDTA solution was added and the flasks incubated at 37^0C for 5-15min until the cell became detached. The cell solution was centrifuged (175g, 5min) and re-suspended in fresh culture medium. For freezing cells, the pellet was resuspended 1ml 10% DMSO/FCS at room temperature instead of normal medium, placed in a 1.6ml cryo-tube (Nunc), step-frozen over 12h and then transferred to long term storage in liquid nitrogen. Frozen cells were thawed rapidly in a 37^0C water bath and transferred into RPMI 1640 (5ml, 20^0C). After centrifugation (175g, 5min) the pellet was resuspended in 2ml of culture medium (10% FCS) and the cells were seeded into a culture dish.

2.2.7   Human respiratory epithelial cell line 16HBE 14o-

This cell line has been derived from the airway epithelium of an 1 year old infant and immortalized by SV40 transformation (Cozens et al., 1994). The cells were propagated as described for A549 cells.

2.2.8   Measurement of cytokines in monocyte/macrophage culture supernatants

The cytokines TNF-α, IL-1 and IL-6 were measured by enzyme-linked immuno-sorbent assay (ELISA) with the monoclonal capture method (Ferrante et al., 1990). Microtiter plates were coated with the IgG fraction of goat anti-mouse IgG. After incubation (18-20h, 4^0C) the respective anti-cytokine monoclonal antibody was added to each well. The plates were incubated (3h, 37^0C) and then dilutions of either cytokine standards or dilutions of unknown sample (supernatants) were added. After a further incubation (18-20h, 4^0C) rabbit anti-cytokine antiserum was added and incubated again (3h, 37^0C). Sheep anti-rabbit immunoglobulin-horseradish peroxidase conjugate (60µl) was added and after further incubation (3h, 37^0C) the wells were washed and ABTS buffer was added. The colour reaction was allowed to proceed for 45-60min and the light absorption read at 410nm in a Dynatech plate reader. The TNF-α assay did not detect IL-1 or IL-6, the IL-1 assay did not detect IL-6 or TNF-α and the IL-6 assay did not detect TNF-α or IL-1.

2.2.9   Lucigenin dependent chemiluminescence assay

Lucigenin-dependent chemiluminescence is a measure of neutrophil superoxide production and was used as described by Hardy et al., 1994. This response is specific for superoxide and can be totally inhibited by superoxide dismutase (Gyllenhammar, 1986; Hennet et a., 1993). For each assay 1x 10^6 neutrophils in 100µl HBSS were transferred into luminometer tubes and then various reagents were added in a final assay volume of 500 µl. The neutrophils were pretreated with TNF and then with LPS

in the presence of 1% heat inactivated (56^0C/30min) autologous donor serum. During the pretreatment times the assays were incubated at 37^0C in an atmosphere of 95% air/5% CO_2 and high humidity. The tubes were then transferred into the luminometer (Model 1251, Bioorbit Oy, Turku, Finland, Photograph 2-4), water jacketed (37^0C) with MultiUse^{TM} software version 1.08), which automatically added 500µl of lucigenin per tube to bring the final volume to 1ml. Recording of the resulting chemiluminescence (in mV) began immediately and was continued until the readings returned to baseline values. The results were recorded as peak initial rate of superoxide production and also as total amount of superoxide produced over a fixed time period by integration of the area under the curve (in mV). Some data are expressed as stimulation indices. To obtain these, the means of the treatments were divided by the means of the baseline values.

Photograph 2-4:   Luminometer Model 1251 from Bioorbit Oy, Turku, Finland. The sample tubes were inserted in a carousel, and the luminometer pump automatically injected 0.5ml of lucigenin per tube. Chemiluminescence (in mV) was continuously recorded.

2.2.10   Cytochrome C assay

This method to assess superoxide release is adapted from Babior *et al.* 1972. Neutrophils (1×10^7 cells in 100µl) were pre-treated with either TNF-α or diluent (20min, 37^0C), then mixed with 200µl of cytochrome C (final concentration 100mM) with or without superoxide dismutase (SOD, 80µg/sample). Then 100µl of LPS or diluent were added to each tube, the volume made up to 1ml with HBSS, and incubated (45min, 37^0C). The tubes were centrifuged (560g, 5min) and the light absorption of the cell-free supernatant was read at 550nm in a DU-65 spectrophotometer (Beckman Instruments Inc., Palo Alto, Ca.). Results from blank tubes were subtracted from the readings of all tubes and the superoxide dismutase-inhibitable fraction of superoxide production was calculated by the formula:

$$\frac{OD_{\text{(without SOD)}} - OD_{\text{(with SOD)}}}{18.5 \text{ (Extinction Coefficient)}} = \text{mMoles of } O_2^-/10^7 \text{ PMN}$$

2.2.11   Neutrophil adhesion assay

This assay was carried out in 96 well ELISA plates, which were coated with autologous plasma (10 %, diluted in HBSS, 200µl per well) and incubated at 37^0C for 30min. The wells were washed 2x with 200µl HBSS and $5x10^5$ PMN per well in 50µl HBSS were added. The neutrophils were incubated with various treatments at a final volume of 200ml per well. Experiments were carried out in quintuplets and conducted in a humidified atmosphere (30min, at 37^0C, 5% CO_2). At the end of the treatment period the contents of the well were discarded and the plates washed 2x with HBSS per well. The cells were stained with Rose Bengal (100µl per well, 0.25 % in PBS, 5min, room temperature) and then washed again 3x with HBSS (200µl per well). Cells were fixed with Ethanol/PBS 1:1 (200µl, 30min, room temperature) and then re-suspended gently prior to reading the absorbance of the wells at 570nm in a Dynatech™ plate-reader. Values from wells containing no cells (blanks) were subtracted from values obtained for treatments.

2.2.12   Flow Cytometry and Immunohistochemistry

2.2.12.1   CD11b expression on monocytes/macrophages

The mononuclear fraction obtained from the Hypaque/Ficoll blood preparation ($1x10^6$ cells per tube, 8-20% of which were monocytes on forward/side scatter) were treated in duplicates as described in the figure legends and then washed in ISOTON II, transferred on ice and blocked with 10 µl of Intragam™ for 15min. Then anti-CD11b-PE-conjugated antibody or PE-conjugated isotype-matched control antibody were added at a dilution of 1:50. The cells were washed twice in 2ml ISOTON II per tube, and then fixed in 1:33 Formaldehyde/ISOTON II, (250µl) and analysed in a Becton-Dickinson FACScan, 10,000 events per tube. On Forward/Sidescatter lymphocytes were gated out and the fluorescence intensity of the monocyte population was measured. The fluorescence values of isotype-matched negative controls were subtracted from fluorescence values of the treatments.

2.2.12.2   Binding of fluorescein-labelled LPS

Neutrophils (1.5×10^6), HUVEC (5×10^4) or A549 cells (5×10^4) were incubated at 37^0C for 20min with either diluent or TNF-α. The tubes were transferred onto ice, centrifuged at 4^0C and then re-suspended in serum (4^0C, 5%, heat inactivated). FITC-labeled LPS (Sigma) was added at a concentration of 8μg/ml and non-labeled LPS in the same concentration to a control tube. After 30min incubation on ice the cells were washed three times at 4^0C and fixed in formaldehyde 1:33 in ISOTON II solution. The fluorescence intensity of the cell population was analyzed by flow cytometry on a FACScan^{TM} (Becton Dickinson, USA, Photograph 2-5). A total of 10,000 cells were counted and the data processed with LYSYS II software.

Photograph 2-5:   Becton Dickinson's FACScan. The channel frequencies were:
   FL1 - Excitation: 495nm;   Emission: 510nm. (FITC)
   FL2 - Excitation: 565nm;   Emission: 575nm. (PE)
   FL3 - Excitation: 470nm;   Emission: 677nm. (7-AAD)

2.2.12.3   Adhesion molecules

Endothelial cells were treated in 6 well plates, and the end of the treatment period the cells were washed with HBSS and enzymatically removed with 0.35ml per well of trypsin/EDTA. The cell suspension was collected into FACS tubes (Becton Dickinson), transferred onto ice, washed with 2ml of ISOTON II solution (4^0C) and then resuspended in 10μl of Intragam$^®$ (15min) and then incubated on ice for 30min with 50μl of primary antibody solution (1:100). After 3 washes with ISOTON II (2ml) the secondary antibody was added. After 30min on ice the cells were washed 3x and fixed with 1:33 formaldehyde in ISOTON II (0.3ml). The fluorescence intensity of the cell population was analyzed by flow cytometry on a FACScan (Becton Dickinson). 10,000 cells per sample were counted and the data were processed using Lysis II or Cellquest software (Becton Dickinson). The

fluorescence values of isotype-matched negative controls were subtracted from fluorescence values of the treatments.

2.2.12.4   Cell surface CD14

Essentially, cells were processed as described above. Some cells were not fixed and re-suspended in ISOTON II for assessment of their viability according to their positive or negative staining with 7-amino-actinomycin D (7-AAD, 0.2%, FL3). After plotting the cell population forward scatter against flow channel 3 (FL3), 10,000 events from within the viable, 7-AAD-unstained cell population per sample were counted. The endothelial cell viability (7-AAD-negative cells) was > 90%. To rule-out artifacts produced by the use of trypsin to remove HUVEC from plastic, gentle mechanical removal with a cell scraper ("rubber policeman") was compared to cells obtained from the same cord. There was no difference in the level of CD14 surface expression or in cell viability between the two procedures (not shown). A representative dot plot of HUVEC stained with the viability stain 7-AAD (FL3) is shown in Fig. 2-1.
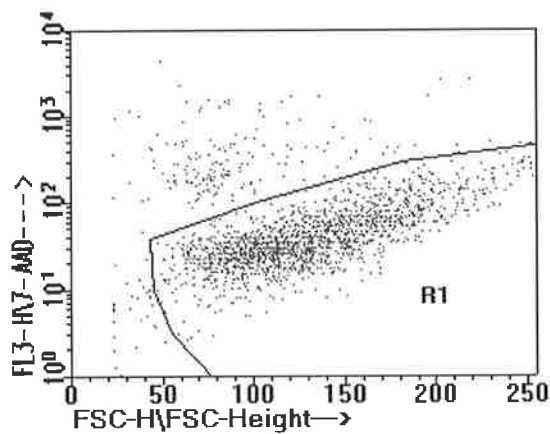
Figure 2-1:   Viability of HUVEC at the time of flow cytometric analysis. After enzymatic removal of untreated HUVEC from the culture dish and standard preparation for flow cytometric analysis the cells were stained with 7-AAD (10min, 0.2%). Gate R1 contains the viable (7-AAD-negative) cells, > 90% of the total cell population of approximately 10,000 cells.

7-AAD can be used together with FITC and PE and stains only cells which lost their membrane integrity (Schmid et al., 1994). The median fluorescence intensity-values (MFI) of isotype-matched negative controls were subtracted from the MFI of samples with anti-CD14. The expression of CD14 on neutrophils, monocytes or epithelial cells was carried out in a similar fashion.

2.2.12.5   Von Willebrand Factor staining of endothelial cells

Staining of HUVEC with rabbit antiserum to human von Willebrand factor was carried out as prescribed in the instructions of DAKOPATTS kits. HUVEC were grown to confluence in 6-well plates. The cells were washed and fixed with methanol (20min) in the presence of H_2O_2 (0.6%) to quench endogenous peroxidase. After blocking with 10% rabbit serum rabbit anti-human von Willebrand IgG was added (dilution 1:500). After 1h DAB solution was applied (5min) and the cells were counter-stained with Mayer's Haematoxilin. HUVEC were 99-100% positive.

2.2.12.6   Immuno-histochemistry

A section of a fresh cord was washed in HBSS (4^0C), the vein and arteries were flushed with HBSS and then with 10% formaldehyde. After fixing the tissue in 10% formaldehyde overnight it was embedded in paraffin and cut with a microtom (Leica 1512, Wetzlar, Germany) at 6μm thickness. After mounting, rehydration and quenching with peroxide a standard 3-step immune histochemistry was performed with DAB as the substrate. Slides were counter stained with haematoxilin and photographed with light microscopy (magnification 10-40x).

2.2.13   Immunofluorescence microscopy of HUVEC in culture and of cord tissue

2.2.13.1   Cells in culture

HUVEC were grown on chamber slides, washed with Phosphate Buffered Saline and fixed (PBS, 37% Formaldehyde and 2% Triton™ X100. After blocking (human immunoglobulin G, 20min) two-step staining was performed with MY4 (1:50) primary antibody and FITC-conjugated rabbit-anti mouse polyclonal secondary antibody (1:100).

2.2.13.2   Cord tissue

A section of cord was snap-frozen in 20% ethanol (-170^0C), cut into 10μm thick sections using a cryomicrotome and transferred onto slides. After air-drying tissue samples were stained as described above. Samples were photographed with a UV fluorescence microscope (Leitz, Wetzlar, Germany) using Kodak 100 ASA slide film.

2.2.14 ELISA for measuring expression of adhesion molecules on HUVEC

HUVEC (5×10^4/well) were plated into 96 well plates, which had been pre-coated with 0.2% gelatin. Cell reached confluence within 1-3 days and were treated in the wells. At the end of any treatment period the wells were washed twice with warm 0.1% BSA in HBSS 0.2ml per well and then fixed overnight with glutaraldehyde (0.025%, 20^0C, 0.2ml/well). The monolayers were then washed twice with BSA and incubated with blocking buffer (0.1%BSA in HBSS and 0.1M glycin, 20^0C, 2h). The ELISA was performed with 3 washes with 0.2ml / well 0.1% BSA between each step. HUVECs were incubated first with 50µl/well of primary monoclonal antibody (anti-E-selectin or anti-ICAM-1 or anti-VCAM-1), and then with 70µl/well of secondary HRP-conjugated antibody, one hour each at 37^0C. Finally, 100µl per well of enzyme substrate consisting of 0.55mg/ml 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate-(6)] and 0.012% hydrogen peroxide in citrate-phosphate buffer, pH 4.2 were added and colour was developed until cell-alone wells gave a standardized absorbance reading at 410nm (optical density, 0.3 units) using an ELISA plate reader (Dynatech MR 7000).

2.2.15 Estimation of adhesion molecule mRNA

The human E-selectin c-DNA probe was a 3.8-kb insert cloned into the *Xho-I* site of a pB-SK vector. After the treatments of HUVEC in tissue culture plates RNA for the slot blots was isolated by the $RNA_{zol}B$ method by direct addition of 0.4ml $RNA_{zol}B$ per 10^6 cells and lysis on the tissue culture plate. Chloroform (50µl) was added to the lysates, each tube was vigorously shaken for 15s and placed on ice for 5min. After centrifugation (9,000g, 15min, 4^0C) the upper phase was carefully removed and the RNA was precipitated by the addition of an equal volume of isopropanolol by incubation on ice for 15min. The RNA was pelleted by centrifugation at 9,000g (15min, 4^0C). The RNA samples were diluted in a solution containing formaldehyde, SSC, SDS, and diethyl-pyrocarbonate and heated (65^0C, 5min). The samples were applied to a Gene Screen Plus nylon membrane using a slot-blot vacuum manifold. The filters were baked (2h, 80^0C), and prehybridization was performed for 16 to 24h in a solution containing formamide, dextran sulfate, SDS, SSC, Denhardt's solution and denatured herring sperm DNA. DNA probes were oligolabeled using [α-^{32}P]dCTP as described previously (10) and used for hybridization. The extent of hybridization was subjected to quantitative analysis by Instant Imager and Imager software (Packard Instruments). The quantitations for the blots were calculated as the value obtained for the E-selectin probing of that blot divided by the value of its GAPDH reprobing and expressed as normalized ratio.

2.2.16   Nuclear extracts

Nuclear extracts were prepared as described previously (Min et al., 1997) with slight modifications. Briefly, confluent endothelial monolayers were stimulated with diluent, TNF, LPS or TNF and LPS. After incubation cells were washed twice with cold PBS (8ml, 4^0C), harvested with a cell-scraper and centrifuged (175g, 5min, 4^0C). The pellet was resuspended in 1ml cold PBS. Following centrifugation (9,000g, 4^0C, 15s) the cells were lysed (10min, 4^0C) by adding 80µl of lysis buffer (pH 7.8, 10mM Hepes, 1.5mM $MgCl_2$, 10mM KCl, 300mM sucrose, 0.5% (v/v) Nonidet P-40, 1mM dithiothreitol, 1mM phenylmethylsulfonyl fluoride (PMSF), 10µg/ml leupeptin, 10µg/ml aprotinin, 10µg/ml pepstatin A, 10µg/ml benzamidine). The tubes were washed a second time, the pellets were resuspended in 60µl lysis buffer and the nuclei disrupted by sonication. After centrifugation (9,000 g, 30s) the supernatants were collected, mixed with Laemmli Buffer (100^0C, 5min) and stored at -70^0C until analyzed by western blotting.

2.2.17   Western Blot

The assay was carried out as described previously (Hii et al., 1998). The proteins were separated by 12% SDS polyacrylamide gel electrophoresis (PAGE). After transfer the blots were incubated with primary antibody, washed (6x) and subsequently incubated with secondary antibody (both antibodies at 1:1000, 1h, 37^0C). After 6 washes immunocomplexes were visualized by enhanced chemiluminescence. The blots were scanned with an Image Quant™ scanner and quantified with Image Quant™ software version 3.3 (Molecular Dynamics, USA).

2.2.18   Assay for the activity of the MAP kinases ERK and p38.

After exposure of HUVEC to the various agonists for 15min incubations were terminated by removing the culture medium and washing the cells twice (8ml HBSS, 4^0C). 200µl of lysis buffer per dish (20mM Hepes, pH 7.4, 0.5% (v/v) Nonidet P-40, 100mM NaCl, 1mM EDTA, 2mM Na_3VO_4, 2mM dithiothreitol, 1mM PMSF, 10µg/ml leupeptin, 10µg/ml aprotinin, 10µg/ml 10µg/ml pepstatin A, 10µg/ml benzamidine) were added and the cells were harvested with a cell scraper and incubated with constant mixing (4^0C, 2 h). After centrifugation (9,000g, 20s) the supernatants were collected and stored at -70^0C until assayed for kinase activity. ERK and p38 were immunoprecipitated before determination of kinase activity as described by Hii et al., 1998. Lysates were precleared with protein A-Sepharose. Anti-p38 or anti-ERK antibody (3µg/sample) was added and tubes were incubated with constant mixing (90min, 4^0C). The antigen-antibody complexes were precipitated by the addition of protein A-Sepharose. The immuno-precipitates were washed twice at 4^0C, first with lysis buffer and

then with assay buffer (20mM Hepes, pH 7.2, 20mM β-glycerophosphate, 3.8mM p-nitrophenylphosphate, 10mM MgCl$_2$, 1mM dithiothreitol, 50μM NaVO$_4$ and 20μM ATP). The assay was started by adding 30μl of assay buffer containing 6 μCi of γ^{32}P-ATP per sample and 35μg/ml of myelin basic protein. After a 20min incubation at 30^{0}C, the assay was terminated by the addition of Laemmli buffer and the samples were boiled (100^{0}C, 5min). Phosphorylated myelin basic protein was resolved by 16% SDS-PAGE and was detected and quantified using an Instant Imager and Imager software (Packard Instruments).

2.2.19 Assay for the activity of the MAP kinase JNK.

JNK was assayed by a solid phase method as described by Hii et al., 1998. Glutathione S-transferase-jun 1-79 fusion protein was purified from bacterial lysates using glutathione-Sepharose 4B at 4^{0}C with gentle rocking. Lysate protein (1000μg), 15mM MgCl$_2$ and 10μM ATP were added to 25μl (packed volume) of glutathione S-transferase-jun (1-79) coupled to glutathione-Sepharose beads. The mixtures were incubated with gentle rocking (2h, 4^{0}C). After centrifugation (9,000g, 4^{0}C, 5min) the beads were washed once with lysis buffer, once with wash buffer (pH 7.0, 10mM Pipes, 100mM NaCl) and once with assay buffer (as above). The assay was initiated by adding 30μl assay buffer containing 6μCi of γ^{32}P-ATP/sample (30^{0}C, 20min) and terminated by the addition of Laemmli buffer (100^{0}C, 5min). Samples were resolved by 12% SDS PAGE and detection and quantification of phosphorylated glutathione S-transferase-jun 1-79 was carried out as above.

2.2.20 Incorporation studies with radio-labelled amino acid

HUVEC were grown to confluence in 10cm tissue culture dishes. Cells were either maintained in normal culture medium (unstimulated), or in medium containing 40% FCS (stimulated). 25μCi of ^{35}S-L-Methionine were added to each dish in a final volume of 10ml. After 24h the cells were washed once on ice, mechanically removed from the dishes, and lysed in constant motion at 4^{0}C for 4h with 250μl of lysis buffer (20mM Hepes, pH 7.4, 0.5% (v/v) Nonidet P-40, 100mM NaCl, 1mM EDTA, 2mM Na$_3$VO$_4$, 2mM dithiothreitol, 1mM PMSF, 10μg/ml leupeptin, 10μg/ml aprotinin, 10μg/ml 10μg/ml pepstatin A, 10μg/ml benzamidine). Triton X 100 at a concentration of 0.5mg per mg sample protein was added and membranes disrupted by sonication. The samples were centrifuged at 100,000g (4^{0}C, 1h) and then transferred onto 15μl each of protein A sepharose beads for preclearance at constant motion (4^{0}C, 45min). Samples were centrifuged (30s, 4^{0}C, 9,000g) and the supernatants transferred into 2 new tubes per sample. Either anti-IgG$_{2b}$ or anti-CD14 antibody (MY4) were added, 12μl per tube. The samples were kept at gentle agitation (4^{0}C, 2h) and were subsequently transferred onto 12μl each of protein A sepharose beads. Again, samples were incubated with gentle agitation (4^{0}C, 1h) and then centrifuged (4^{0}C, 9,000g). The supernatant was discarded and the beads

resuspended in scintillation fluid (6ml) and placed into scintillation vials. Radiation as counts per minute (CPM) was recorded in a β-counter (LKB Wallac 1409-411 Liquid Scintillation counter, Turku, Finland) over a period of 10min per sample, the result of the IgG_{2b} control precipitations were subtracted from the results obtained with MY4.

2.2.21   Lowry's Protein determination

Protein standards are prepared by serially diluting 0.1% BSA 1:2 in tubes including a tube containing H_2O only (zero protein). A 1:10 dilution of each sample to be assayed is produced (5µl in 45µl H_2O), and Solution I (150µl of Lowry's Solution/CuSO2 0.1M, 100:1) is added to all tubes. After incubation (room temperature, 20min) Solution II (15µl of H_2O and Folin's reagent, 1:1) is added. After incubation (room temperature, 30min) the contents of all tubes are transferred into a 96-well plate (180µl/well) and the light absorption is determined in a plate reader (560nm, Dynatech MR7000). The resulting optical density (OD) readings of the known protein standards are plotted on a linear regression normogram. This allows the protein content of the treatment samples to be calculated by comparing their OD values to the normogram.

2.3   STATISTICAL ANALYSIS

Data are represented as means ± standard errors of the mean unless stated otherwise in the text or the figure legends. Statistical significance was assessed either by Student t test when comparing two groups of data or by analysis of variance (ANOVA) for multiple comparisons. If data were not normally distributed the nonparametric Kruskal-Wallis test with Dunn's post-test was used. P values of less than 0.05 were considered statistically significant.

Chapter 3

Responses of Mononuclear Phagocytes
to LPS and Pyrogenic Cytokines

3.1   INTRODUCTION

Peripheral blood monocytes and tissue macrophages are an important part of the innate immune system. These cells play many different roles in defense against infection, with one of their major functions being phagocytosis of foreign matter. Therefore monocytes and macrophages are often referred to as 'professional phagocytes'. Another important role for macrophages is processing of antigen and the release of cytokines, which promote T-cell activation.

It is also well known that the activity of macrophages can be dramatically increased by a variety of endogenous and exogenous mediators. In relation to many extracellular bacteria short-term stimulation of macrophages with cytokines increases their bactericidal activity. In other cases as for intracellular bacteria like mycobacteria and listeria, macrophage activation is required for the cell to control and kill the intracellular organism, a process which requires long-term exposure to specific mediators such as IFN-γ.

During inflammation, initiated by microbial invasion of tissues, mononuclear phagocytes become simultaneously exposed to a mixture of mediators which all function to alter the antimicrobial activity of the cell (Henderson et al., 1996). This manifests itself in terms of increased expression of cell surface receptors which aid the cell's ability to recognize bacteria and tissue surfaces, in terms of initiation of the oxygen-dependent respiratory burst as well as the release of lysosomal enzymes and other mediators (Frenette et al., 1996; Cassatella et al., 1989). Mononuclear phagocytes also produce and release cytokines which inhibit the growth or kill/damage microorganisms, tumour cells and altered tissues (Ferrante et al., 1992).

To appreciate the mediator network, which orchestrates the monocytes response to infection there is a need to address the issue of changes associated with exposure of monocytes to more than one mediator. The main reports so far have addressed the question of the effects of one mediator on the function of the mononuclear phagocyte.

The purpose of the investigations described in this Chapter was to gain further understanding of modification of mononuclear phagocyte function by mediators by examining whether concomitant exposure to more than one mediator resulted in significant changes in cell function compared to the effect of either mediator alone. In this study the endogenous pyrogenic cytokines TNF-α, IL-1β and IL-6, and the bacterial product, LPS, were chosen since these are the main mediators encountered in the scenario of gram-negative infections. LPS is the most powerful inducer of the synthesis of cytokines by monocytes, including TNF-α, IL-1 and IL-6. The other marker of monocyte stimulation studied was the surface expression of the adhesion molecule CD11b/CD18 (CR3), the level of which correlates with the adhesive properties of the mononuclear phagocyte.

3.2 THE PRODUCTION OF TNF-α , IL-1β AND IL-6 BY LPS-STIMULATED MONOCYTES

Monocytes were prepared from the peripheral blood of healthy volunteers by a combination of density gradient centrifugation and adherence to serum-coated plastic surfaces (as described in methods, section 2.2.1). The monocytes (1×10^6) were treated in $2cm^2$ wells (final volume 500μl) with varying concentrations of LPS in HBSS containing 5% autologous donor serum and incubated at 37^0C. The supernatants were collected after 6 h and assayed for TNF-α, IL-1β and IL-6 by ELISA (described in 2.2.8). The results showed, that the amount of TNF-α, IL-1β and IL-6 produced was LPS concentration-dependent. Significant amounts of TNF-α were produced with LPS concentrations of 0.2ng/ml, while IL-1β and Il-6 production required only 0.02ng/ml of LPS (Fig 3-1).
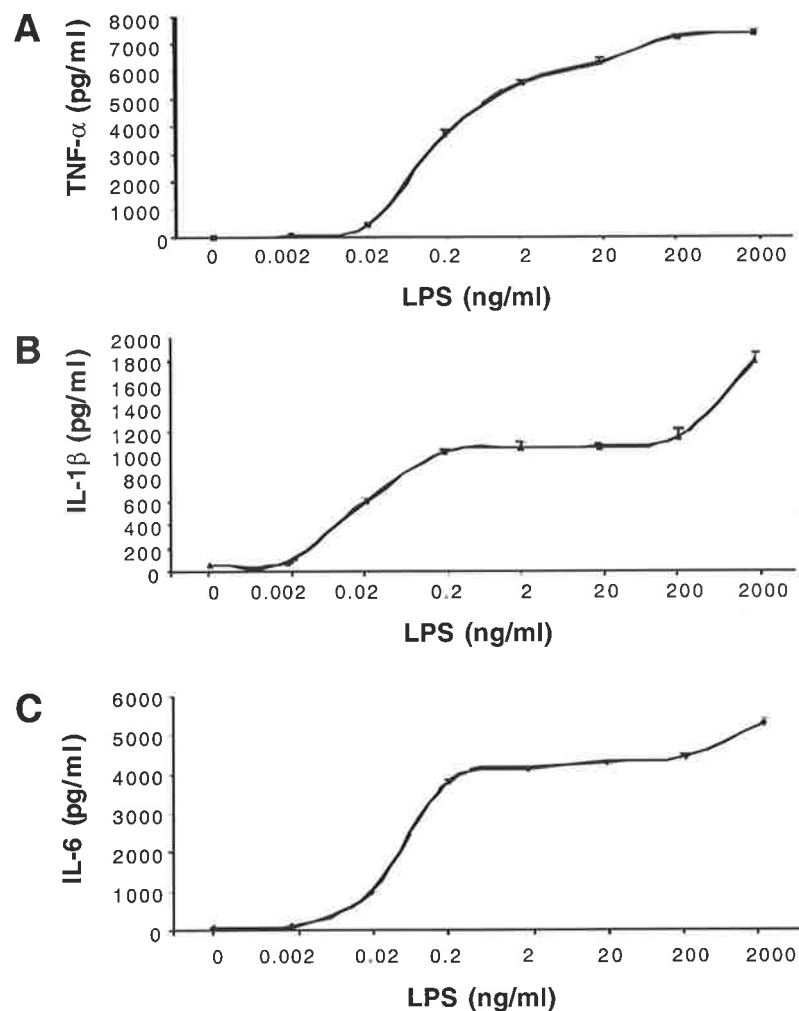
Figure 3-1:   Dose-response curves of LPS-induced production of TNF-α, IL-1β and IL-6 by monocytes. Peripheral blood monocytes (1×10^6cells/assay, volume 500μl) were stimulated for 6h with the LPS concentrations indicated. The data show the means ± SEM of 2 determinations with cells from two different individuals. The results are expressed as amounts of cytokine produced (in pg/ml).

3.3 THE EFFECT OF TNF-α ON THE LPS-INDUCED IL-1β PRODUCTION

TNF-α produced in response to LPS by monocytes and macrophages is likely to act in concert with LPS to produce a cascade of inflammatory-associated responses. To examine this concept peripheral blood monocytes were treated with either TNF-α (10U/ml), LPS (0.1ng/ml) or both agonists. The amounts of TNF-α and IL-1β produced by monocytes exposed to both mediators were not only greater than the amounts induced by each agonist alone, but were also greater than the sum of the responses by the individual treatments, suggesting that the two mediators were synergistic in this response (Fig. 3-2).
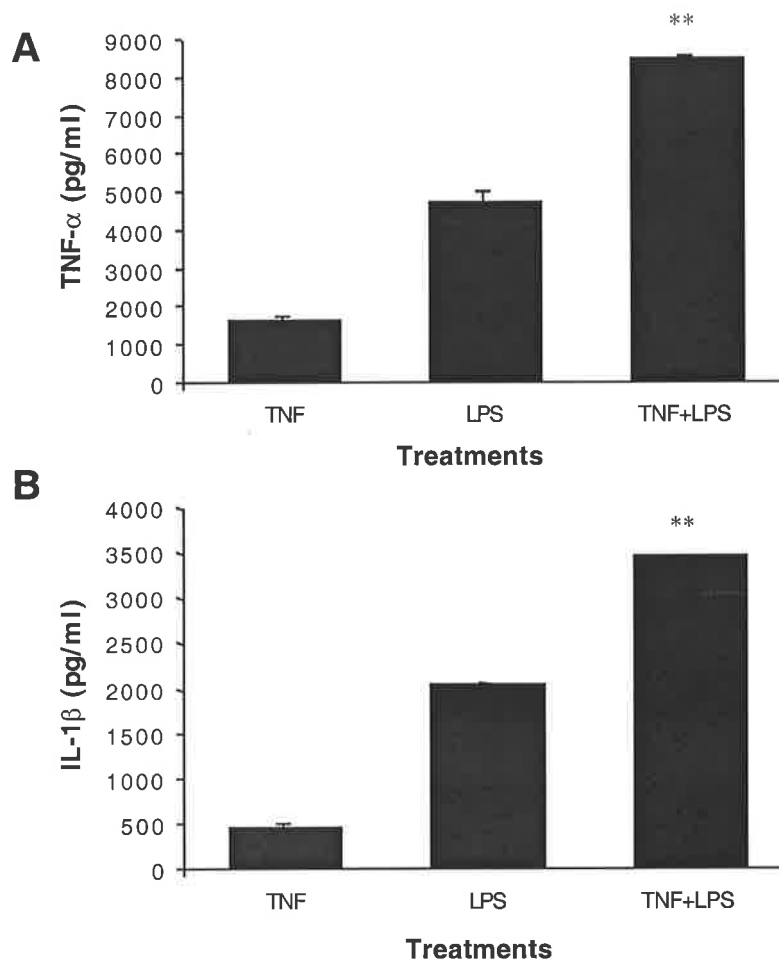
Figure 3-2:   Effect of TNF-α on LPS-induced production of TNF-α (**A**) and IL-1β (**B**). Peripheral blood monocytes (1x10⁶cells/assay) were treated for 3h with either diluent, TNF-α (10U/ml), LPS (0.1ng/ml) or both agonists combined (TNF+LPS) in the presence of human serum (2%, final assay volume 1ml). The results are expressed as the means ± SEM of two experiments with cells from 2 different donors. The values for untreated cells (**A**, TNF-α, 109pg/ml; **B**, IL-1β, 89pg/ml) have been subtracted from the treatments. The amount of cytokine produced was measured as described in methods.   ** p < 0.001 for TNF+LPS vs TNF or LPS. p < 0.001 for TNF+LPS vs sum of TNF and LPS in (**A**) and p < 0.01 for TNF+LPS vs sum of TNF and LPS in (**B**)

3.4 THE EFFECT OF TNF-α, IL-1β AND LPS ON MONOCYTE IL-6 PRODUCTION

The cytokine IL-6 is produced by monocytes and macrophages not only in response to LPS, but also to TNF-α and IL-β. The hypothesis that all three mediators may cooperate to increase IL-6 production, was tested by stimulating peripheral blood monocytes (1×10^6/ml) with TNF-α (10U/ml), LPS (0.1ng/ml), IL-1β (1ng/ml), each alone or in a number of combinations of these. For all combinations there was a statistically significant increase in the production of IL-6 (in pg/ml) when compared to the amount of IL-6 generated by each agonist individually (Fig. 3-3). When combined TNF-α and LPS (Fig. 3-3A), TNF-α and IL-1 (Fig. 3-3B) or LPS and IL-1 (Fig, 3-3C) achieved a moderate but statistically significant increase in IL-6 production. An additional increase in IL-6 production was achieved when monocytes were treated with the combination of all three mediators (Fig. 3-3D).
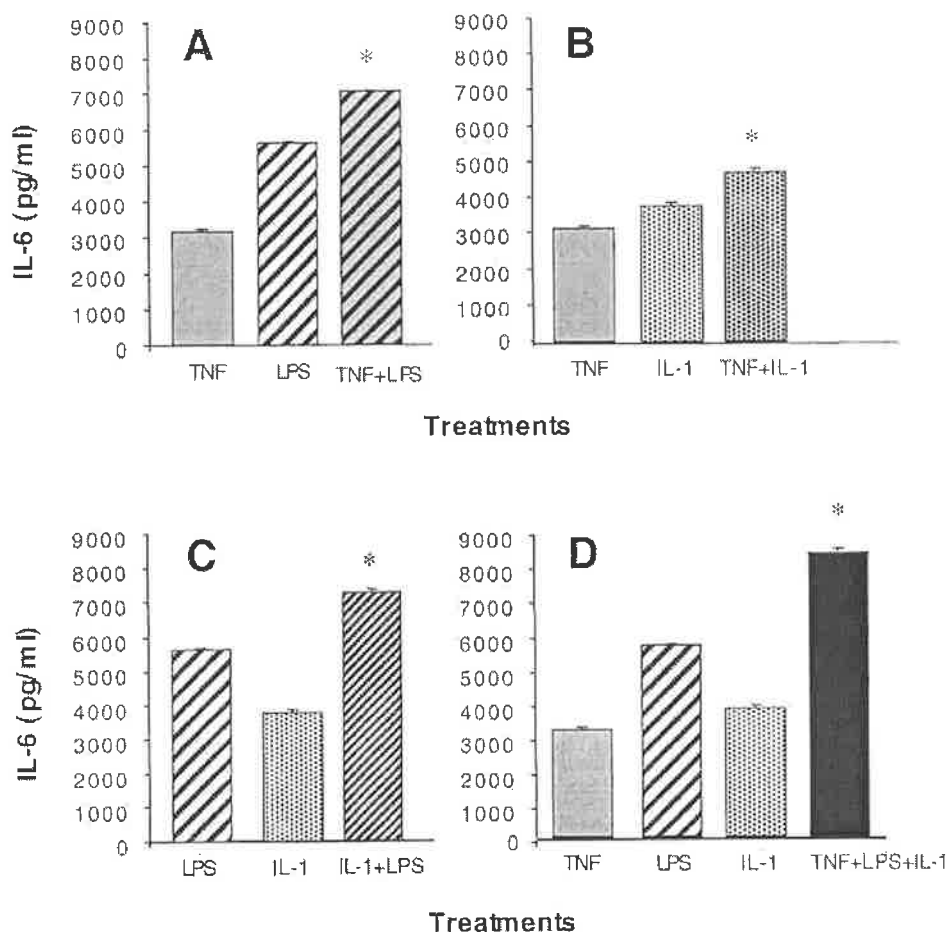
Figure 3-3:   Effect of TNF-α, LPS and IL-1β on the production of IL-6 by monocytes. Peripheral blood monocytes (1×10^6 cells, assay volume 1ml) were treated for 3h with either TNF-α (10U/ml), LPS (0.1ng/ml), IL-1β (1ng/ml) or combinations as indicated. The amount of IL-6 produced was determined by ELISA and comparison with known standards. The data represent the means ± SEM of 2 determinations of 2 independent experiments. The amount of IL-6 produced by unstimulated cells (control) was 320 pg/ml and has been subtracted from all treatment values. (* p < 0.001 for the combinations vs each of the single agonists.)

3.5 EFFECT OF TNF-α ON THE LPS-INDUCED PRODUCTION OF TNF-α BY MACROPHAGES

To examine whether macrophages showed a similar response pattern to monocytes (Fig. 3-2A), peripheral blood monocytes were differentiated into macrophages in serum-coated tissue culture plates. The treatment conditions were the same as for monocytes. The production of TNF-α by macrophages was synergistically increased when cells were treated with LPS and TNF-α (Fig. 3-4).
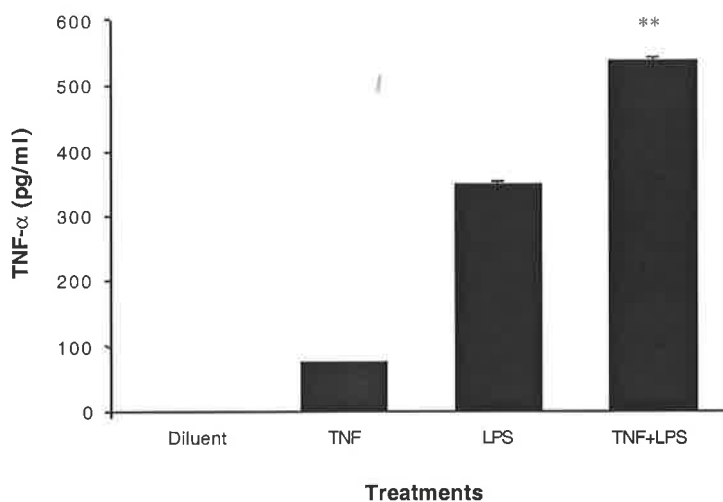
Figure 3-4:   Effect of human recombinant TNF-α on the LPS-induced production of TNF-α by macrophages. Peripheral blood monocytes were differentiated into macrophages as described in Methods. Macrophages were treated in the 6-well plates they differentiated in without being handled. Cells (1×10^6/assay were treated with either diluent, TNF-α (10U/ml), LPS (0.1ng/ml), or both for 3h. The entire treatment volume (1ml) was collected for cytokine measurements. The data represent the means ± SEM of 2 separate experiments with monocytes from 2 donors. ** $p < 0.001$ for TNF or LPS vs TNF+LPS; $p < 0.01$ for the sum of TNF and LPS vs TNF+LPS.

3.6 EFFECT OF TNF-α ON LPS-INDUCED UPREGULATION OF CD11b/CD18

It has been reported that LPS and TNF-α each have the ability to upregulate the expression of the β-integrin family adhesion molecule CD11b/CD18. Upregulation of this molecule is associated with increased adhesiveness of mononuclear phagocytes, and CD18 expression correlates with diapedesis of these cells through endothelial monolayers. Thus CD11b/CD18 can be viewed as a marker of cell stimulation, and it was of interest to investigate whether low doses of LPS and TNF-α would synergize in terms of upregulation of this molecule. Monocytes prepared from peripheral blood (1×10^6cells/assay) were treated with diluent, different concentrations of LPS and TNF-α individually, or both mediators combined. Expression of CD11b/CD18 was measured by direct immunofluorescence and flow cytometric analysis of formaldehyde-fixed cells. The data, shown as percent increase over control, demonstrated increased expression of CD11b/CD18 in a dose-dependent manner (Fig. 3-5, only CD11b is shown). The effect of combined treatment with both mediators was additive. However, the overall increase was only modest, the maximum achieved with combined treatment with the largest doses of LPS and TNF-α was 60%.
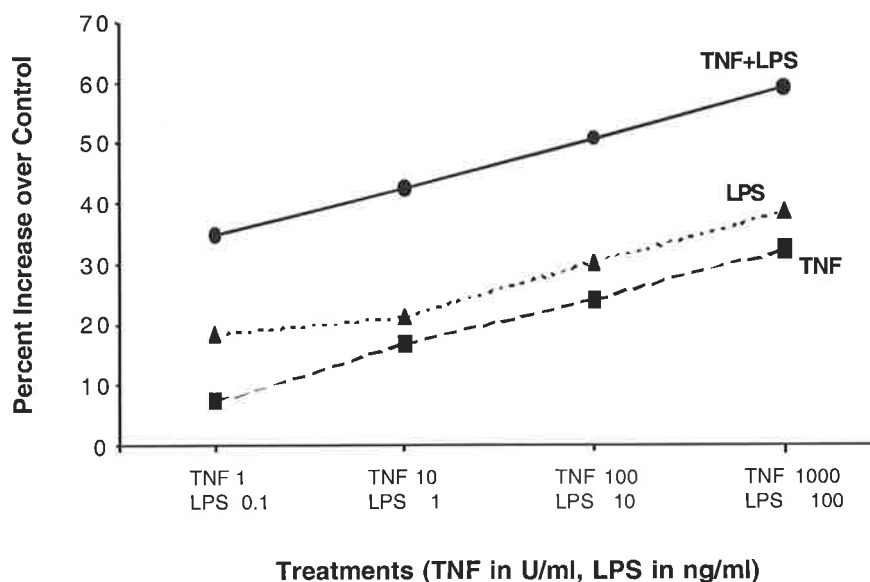
Figure 3-5:   CD11b expression in monocytes. $1x10^6$ monocytes were stimulated with TNF-α or LPS or TNF-α + LPS for 30min at the concentrations indicated. Cells were then stained with an anti-CD11b PE-conjugated antibody and 10,000 cells were analyzed using a FACScan. The results represent the means SEM of duplicates of experiments with cells from 3 different donors and are expressed as percent increase of the median fluorescence intensity (MFI) above the MFI of the unstimulated cell population. (p <0.05 for TNF+LPS vs TNF or LPS at all 4 concentrations tested)

3.7 SUMMARY AND CONCLUSIONS

Suboptimal concentrations of bacterial lipopolysaccharide, as found during a gram-negative bacterial infection, were able to stimulate monocytes to produce TNF-α, IL-1β and IL-6 and synergize with TNF-α to enhance the production of TNF-α and IL-1β significantly .These pro-inflammatory cytokines stimulated the production of IL-6 by monocytes, which was further increased in the presence of LPS. As in monocytes, TNF-α acted synergistically with LPS to stimulate TNF-α production in macrophages, which were derived by differentiating monocytes in culture. Monocytes also showed increased expression of the adhesion molecule CD11b/CD18 if treated with both LPS and TNF-α. The potential limitation of studies of mononuclear cells are cell activation caused by the isolation procedure. The indicator of such activation is a high level of cytokines produced by untreated 'control' cells. In the experiments described above the baseline was very low and the ability of the cells to respond normally to stimulation was confirmed by dose-response studies (Fig 3-1). The data indicate that significant amounts of pro-inflammatory cytokines were released by mononuclear phagocytes when these cells were exposed to low concentrations of both LPS and TNF-α in concert. This is likely to amplify the immune response to bacteria by the activation of local cells and the recruitment of other inflammatory leukocytes.

Chapter 4

The Effect of TNF-α and LPS

on Neutrophil Responses

4.1 INTRODUCTION

Neutrophils differentiate from bone marrow stem cells and are the first line of defense against bacterial invasion of the host. The cells are attracted to relevant locations by chemokines secreted by macrophages and other local cells. Binding to the endothelium is necessary for neutrophils to become rapidly deployed at sites of infection, where they are stimulated to kill microorganisms. This is achieved by an increased production of oxygen radicals and the release of granule constituents, a powerful anti-microbial system (Babior et al., 1972; Sengeløv et al., 1995). However, in exacerbated inflammation persistence of activated neutrophils usually leads to damage of tissues.

Adhesion of neutrophils to endothelium, release of granule constituents and production of oxygen radicals are under the control of endogenous and exogenous mediators. Using TNF-α and LPS as respective examples of stimulators of the above neutrophil functions it was considered important to determine how neutrophils respond to a combination of these two mediators.

4.2 THE EFFECTS OF TNF-α AND LPS ON THE NEUTROPHIL RESPIRATORY BURST

Chemiluminescence assays as a measurement of the neutrophil respiratory burst are based on the fact that the superoxide ion is unstable and activates fluorescent substances, such as luminol or lucigenin, during its dissociation. The resulting measurements are accurate reflections of the rate of superoxide production and are recorded over time, allowing an assessment of peak and of total superoxide production by integration of the data.

LPS directly and strongly triggers the release of ODRS by neutrophils (Dahinden et al., 1983; Nathan, 1987). Although TNF-α has been regarded as being unable to elicit the respiratory burst in neutrophils, recent evidence challenges that view pointing to conditions of cell preparation as the probable reason for these discordant findings. Using the single-step preparation of neutrophils without hypotonic lysis of red cells (as described in Methods), stimulating neutrophils with human recombinant TNF-α and measuring the response with the lucigenin-dependent chemiluminescence in our laboratory TNF-α consistently increased the respiratory burst of neutrophils 2-3 fold (Fig.4-1).
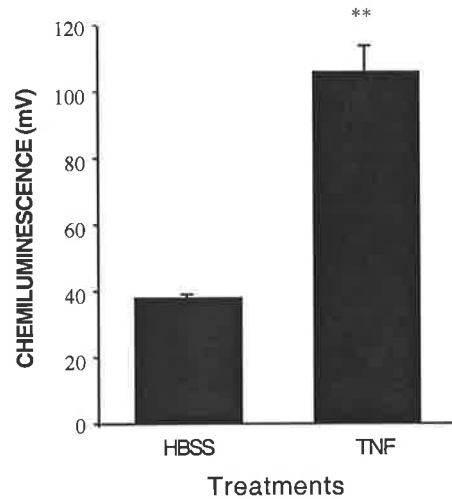
Figure 4-1:   Respiratory burst of neutrophils in response to TNF-α. Neutrophils (10^6cells) were resuspended in HBSS and treated with TNF-α (100U/ml, 37^0C, 30min). The peak initial rate of ODRS production (Chemiluminescence, measured in mV) was 2.8 -fold higher with TNF-α than with HBSS (** p < 0.001). The data represent the means ± SEM of 5 experiments conducted in duplicates.

Since the experiments to be conducted with LPS required the presence of serum, the effect of serum on the TNF-α response of neutrophil chemiluminescence was examined. Serum alone (heat-inactivated, 5%) did not activate neutrophils, however, serum enhanced the neutrophil response to TNF-α by three-fold (Fig. 4-2).
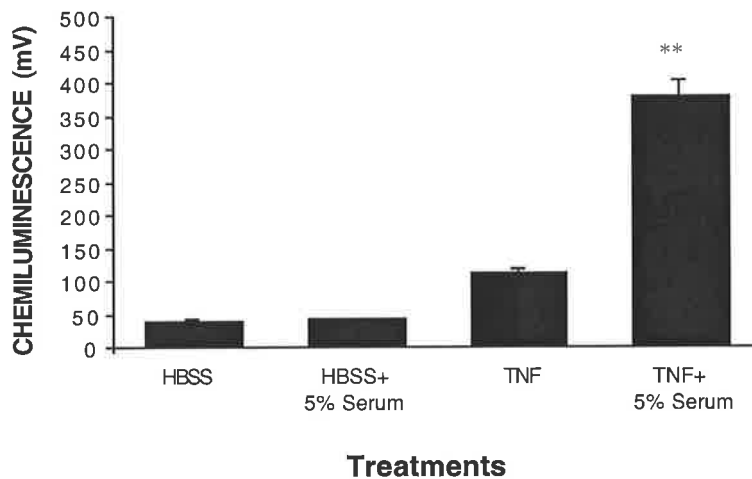
Figure 4-2:   The effect of serum on the TNF-α -induced neutrophil respiratory burst. The autologous donor serum was heat-inactivated at 56^0C for 30min and added to the assays at a concentration of 5% (final assay volume 1ml). The data are shown are the means ± SEM of duplicates of 3 experiments with different cells. The respiratory burst induced in neutrophils ($1x10^6$cells) by 100U/ml of TNF-α was increased 3 -fold in the presence of serum (** p < 0.001).

The influence of serum on the TNF-α response was dose-dependent and the characteristics of this response are shown in Fig. 4-3A. Also, as expected, there was a dose-dependent increase in LPS-induced neutrophil chemiluminescence (Fig. 4-3B).
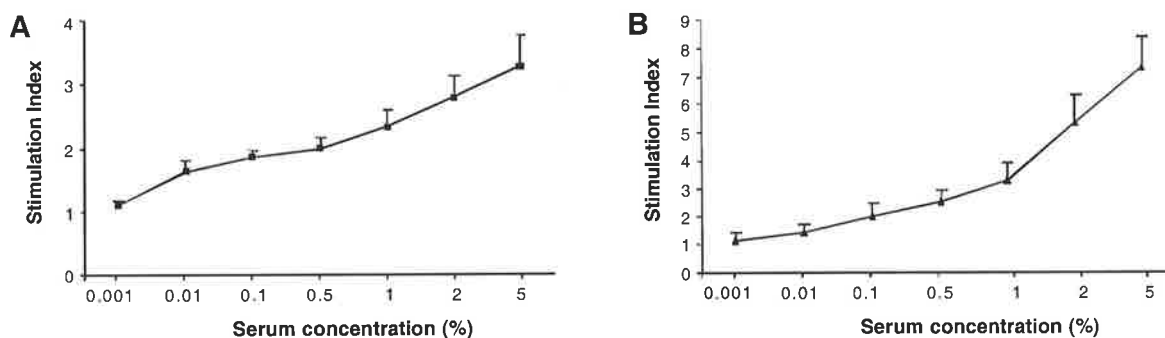
Figure 4-3:   Effect of varying the serum concentration on the TNF-α- and LPS-induced neutrophil respiratory burst. **A.** Neutrophils (1×10^6 cells/ml) were stimulated with TNF-α (100U/ml, 37^0C, 30min) in the presence of the serum concentrations indicated. The data represent the means ± SEM of experiments each carried out with cells from 5 different donors, carried out in duplicates. The data are expressed as stimulation indices, obtained by dividing the means of the treatments by the means of the baseline values (cells treated with HBSS only). **B.** Under the same conditions as in **A.** Neutrophils were stimulated with LPS (1µg/ml, 37^0C, 50min).

In order to characterize the interaction between TNF-α and LPS in terms of enhancement of the neutrophil respiratory burst, different parameters needed to be examined such as the effect of TNF-α pretreatment time as well as the effects at different agonist concentrations.

4.3 EFFECT OF VARYING THE PRETREATMENT TIME WITH TNF-α

Neutrophils (1×10^6) were incubated with TNF-α (20U/ml) for various periods of time over 90min. in an atmosphere of $5\%CO_2$ and high humidity. At the end of the pretreatment time autologous donor serum (heat inactivated, 1%) was added and the neutrophils were incubated for a further 50min with LPS (100ng/ml). The cells were transferred into the luminometer where lucigenin (0.5ml/sample) was automatically injected and the rate of superoxide production was continuously recorded. The optimal pretreatment time with TNF-α was found to be 20-30min (Fig 4-4).
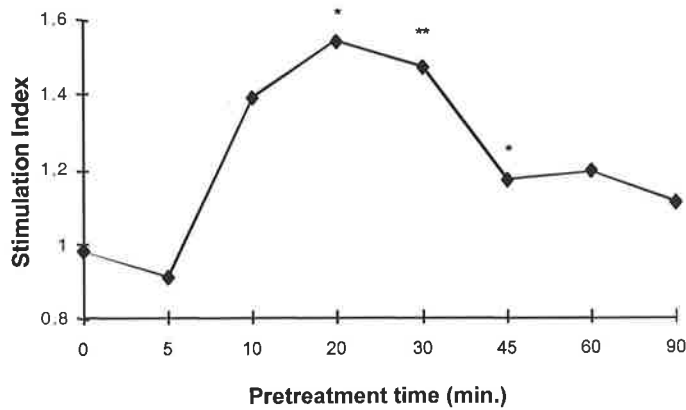
Figure 4-4: The effect of varying the TNF-α pre-treatment times on the response of neutrophils to LPS (*E. coli* K-235). The cells (1×10^6) were pre-treated with TNF-α (20 U/ml) for 20min, followed by treatment with LPS (100ng/ml) for 50min. The results are derived from 8 experiments using different donor cells. The results are expressed as stimulation indices. (* p < 0.035, ** p < 0.015)

4.4 THE TIME CHARACTERISTICS OF THE LPS-INDUCED RESPIRATORY BURST

Published data show that the maximal superoxide production by neutrophils in response to LPS occurs after 45-60min of stimulation. This was confirmed by adding LPS (100ng/ml) to neutrophils (1×10^6 cells) in the presence of serum (1%), placing the cells into the luminometer and continuously recording the chemiluminescence (CL) generated. The maximal response occurred after 50min (±10min, analysis of 10 experiments). (Fig. 4-5). Thus, in subsequent experiments neutrophils were exposed to LPS for 50min.
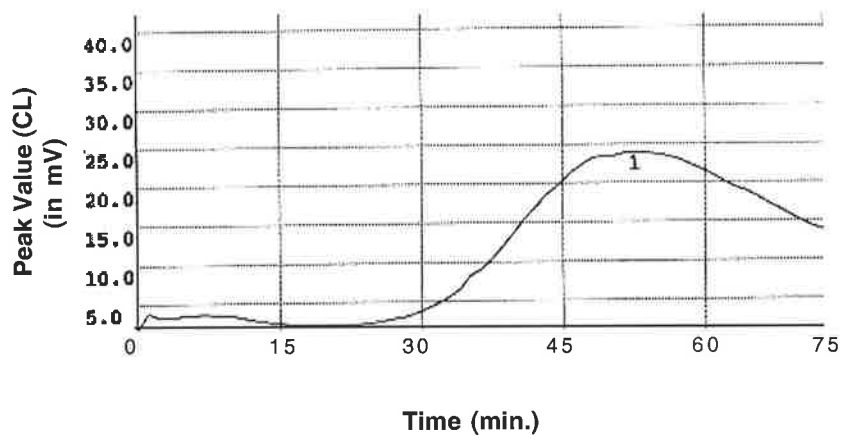
Figure 4-5: Time characteristics of the respiratory burst induced by LPS. LPS (100ng/ml) in 1% serum was added to neutrophils (1×10^6 cells) in a final assay volume of 0.5ml. The cells were transferred into the luminometer and CL was continuously measured for 75min. One representative graph of one of the 10 experiments conducted is shown. The peak-value of the CL baseline (unstimulated cells) was 5.0 (not shown).

4.5 ENHANCEMENT OF THE LPS-INDUCED NEUTROPHIL OXYGEN RADICAL PRODUCTION BY TNF-α

Neutrophils were incubated with HBSS of TNF-α (20U/ml) for 20min. Serum (1%) was added and the cells were incubated with either HBSS or LPS (100ng/ml) for 50min. The treatment volume prior to the injection of 0.5ml of lucigenin was 0.5ml with a total assay volume of 1ml. At completion of the treatment the cells were transferred into the luminometer and the peak rate of CL as well as the total CL accumulated over the incubation period were measured. TNF-α caused a significant increase in the LPS-induced chemiluminescence, both in peak rate and total CL (Fig. 4-6).
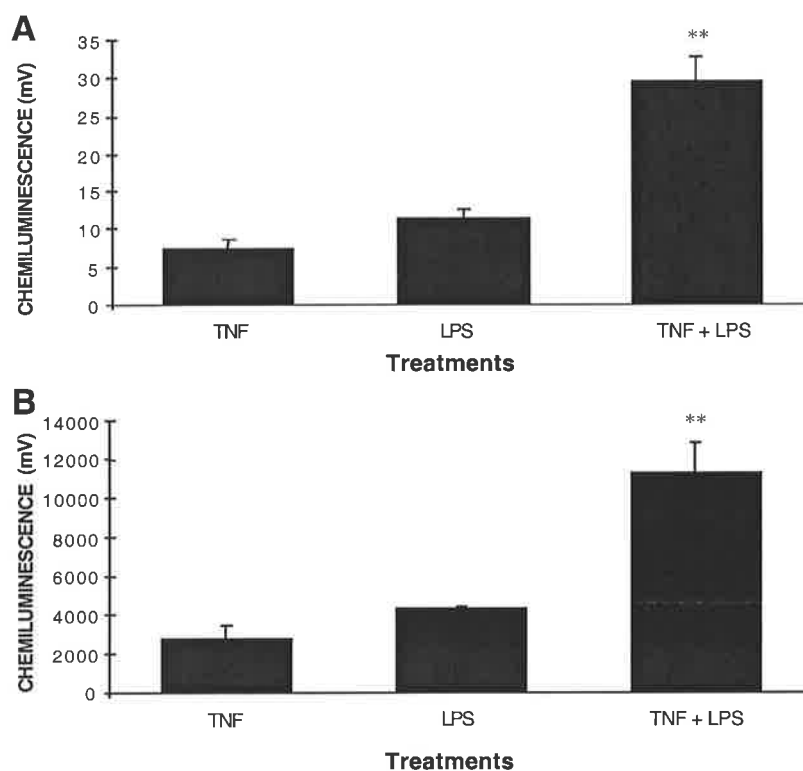
Figure 4-6:   The effect of TNF-α on the LPS-induced neutrophil CL response. Neutrophils (1×10^6) were incubated with 20Units of TNF for 20min. and then challenged with 100ng/ml of LPS (*E. coli* K-235, 50min) and the CL measured. **A.** The values are expressed as the peak initial rate of chemiluminescence and represent the means ± SEM of 8 experiments, each conducted in duplicate with neutrophils from 8 different donors. The basal CL (6.1mV) has been deducted from each of the experimental values. Neutrophils treated with TNF-α and LPS combined showed a significantly increased response compared to responses induced by either of the agents alone **B.** Data are expressed as means ± SEM of the total accumulated CL generated over the incubation period. The baseline value of 2528 mV has been subtracted from each column. (** = $p < 0.001$ for TNF vs TNF+LPS , LPS vs TNF+LPS ; $p = <0.015$ for the sum of values for the individual TNF and LPS treatments vs co-treatment with TNF+LPS).

Based on the effect of the presence of serum on the TNF-induced respiratory burst (Fig. 4-1 and Fig. 4-2), the experiments described above under optimal conditions were repeated with serum present prior to the addition of TNF-α. Interestingly, there was no difference in the magnitude of priming

whether serum (1%) was added to the assay after treatment with TNF-α, prior to the addition of LPS, or at the beginning of the treatment periods, prior to the addition of TNF-α (data not shown).

The effects of different doses of TNF-α on the response to LPS were examined. At all doses of TNF-α (1,5,10,20,100U/ml) there was a significant enhancement of the LPS-induced CL (Fig. 4-7A). A wide range of LPS concentrations, from 0.01 to 1,000ng/ml, was examined, and significant enhancement of the respiratory burst was observed from 1-1,000ng/ml, with the optimal effect observed at 100ng/ml (Fig. 4-7B).
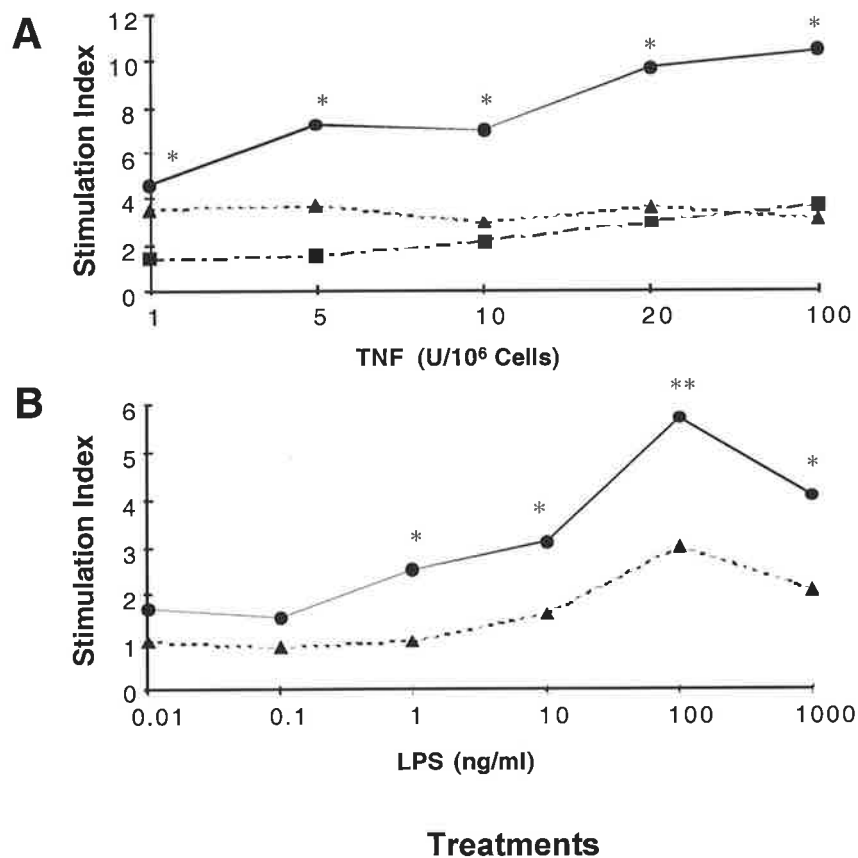
Figure 4-7:   The effects of varying the amounts of TNF-α on the LPS-induced chemiluminescence response. **A.** Neutrophils (1x10⁶cells) were pre-treated with the indicated concentrations of TNF-α or diluent for 20min and then challenged with 1μg/ml of LPS (*E. coli* K-235) or diluent for another 50min. Key: Neutrophils were treated with TNF-α only (■), LPS only (▲) and TNF+LPS (●). Results are expressed as the means of 4 experiments, conducted in duplicates and expressed as stimulation indices. At all TNF-α doses the effects of the combined action of TNF- α and LPS were significantly different from those of either agonist alone (p-values from < 0.001 to < 0.05). **B.** Response of TNF-α-primed neutrophils to varying concentrations of LPS (*E. coli* K-235). The cells (1x 10⁶) were pre-treated with TNF-α (20U/ml) for 20min (●) or diluent (▲) and then stimulated with the indicated concentrations of LPS. Values are expressed as stimulation indices and represent the means of 4 experiments conducted in duplicates   (* p < 0.05, ** p < 0.01).

As an alternative to CL as a method to assess superoxide release by neutrophils the reduction of ferricytochrome C can be measured. This assay is based on the principle that the pigment ferricytochrome C changes its light absorbing properties as it is reduced by superoxide.

$$O_2^{-\cdot} + Fe^{3+} \text{ cytochrome C } \rightarrow Fe^{2+} \text{ cytochrome C } + O2$$
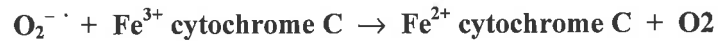
The magnitude of this change can be quantified by spectrophotometry. Tubes are run concurrently to samples containing the same cells and reagents but also superoxide dismutase (SOD). Their results are subtracted from the results of the tube without SOD to determine accurately the proportion of ferricytochrome C reduction that is SOD inhibitable. A potential shortcoming of the cytochrome C assay is that some $O_2^{-\cdot}$ molecules spontaneously dismutase into H_2O_2, which can re-oxidize ferrocytochrome C to its ferric form, hence underestimating the total amount of superoxide produced. Unlike the lucigenin-dependent chemiluminescence the cytochrome C assay only measures the total superoxide production and does not allow assessment of the rate of superoxide ions formed. It is, however, a classic method and regarded as highly reproducible. Therefore neutrophils from 5 different donors were treated as described in section 4.9 and then subjected to the cytochrome C assay, to compare the results with those obtained by lucigenin-dependent chemiluminescence. The experiments were performed with neutrophils from the same donors on the same days and under the same conditions as for the CL-studies. The results are expressed as μmoles of $O_2^{-\cdot}$ per 1×10^7 neutrophils and were similar to the results achieved with chemiluminescence, hence confirming the validity of that method in terms of estimating total superoxide production (Fig. 4-8). To illustrate the importance of the pre-treatment time with TNF-α some cells had TNF-α and LPS added simultaneously at the outset of the experiment.
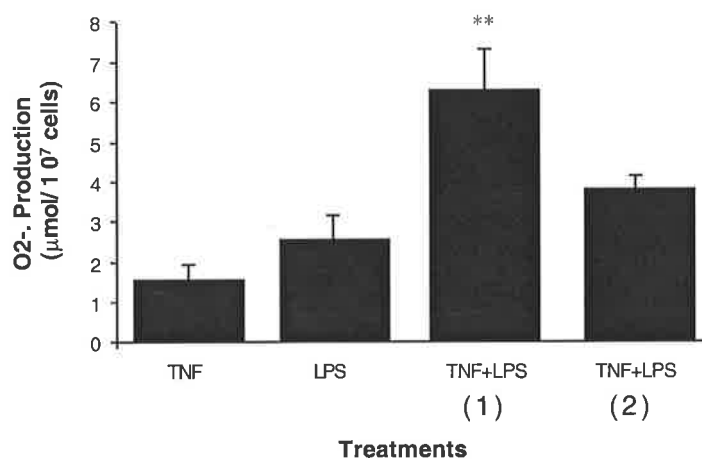
Figure 4-8:   Effect of TNF-α on the superoxide production as measured by the cytochrome C reduction assay. Neutrophils (1×10^7 cells) from 5 different donors were treated under the same experimental conditions as in Fig. 4-6. The absorption of light at 550nm was measured and the results of samples concurrently incubated with SOD subtracted from the samples without SOD. The production of $O_2^{-\cdot}$ was determined as outlined in Methods. TNF+LPS (1), cells were treated with TNF-α for 20min and then LPS was added. TNF+LPS (2), TNF-α and LPS were added to the cells at the same time at the outset of the experiment. (** $p < 0.01$)

4.6 STUDIES WITH LIPOPOLYSACCHARIDES FROM DIFFERENT SEROTYPES OF E.COLI

To ensure that the effects observed were not restricted to a specific *E.coli* strain the experiments were repeated with LPS from *E.coli* serotypes 0127:B8 and 055:B5. The experimental conditions were identical to those described in Fig. 4-6. Neutrophils from 3 different donors were examined and the experiments were conducted in duplicates. Similar to those obtained with serotype K-235, these LPS serotypes were both synergistic with TNF-α for the neutrophil chemiluminescence response (Fig. 4-9).
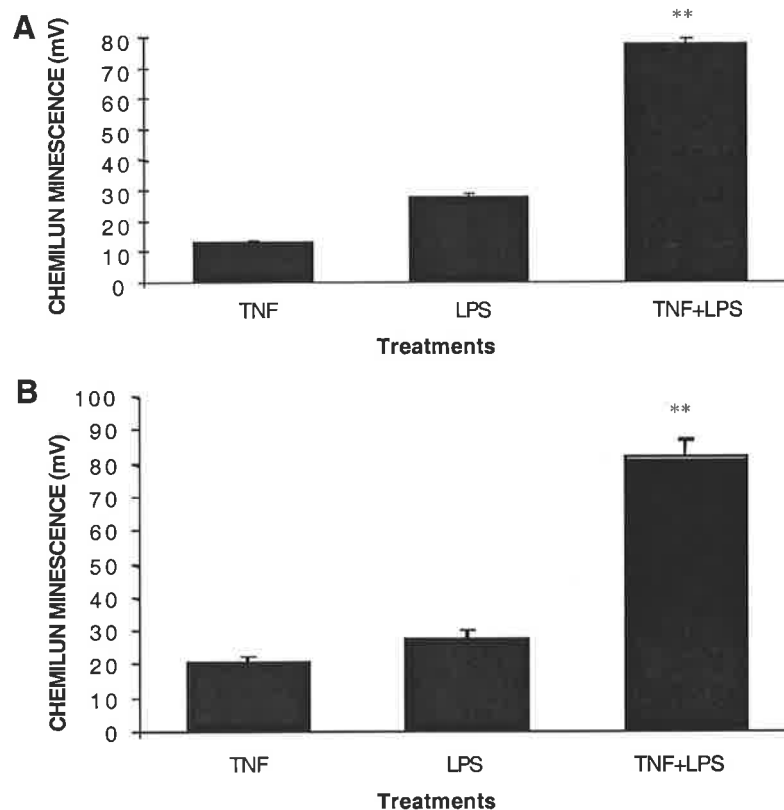
Figure 4-9: Effect of TNF-α on the neutrophil CL response to LPS from other *E.coli* serotypes under the same conditions as in Figure 4-7. **A.** LPS (*E. coli* 0127:B8). The basal CL was 13.3mV and has been deducted from each of the experimental values. **B.** LPS (*E.coli* 055:B5). The basal CL was 9.9mV and has been deducted from each of the experimental values. The experiments were conducted in duplicates with neutrophils from 3 different donors. In both, **A.** and **B.**, the increased response to stimulation with both TNF and LPS was highly significant when compared to either of the single agonists alone (** = p < 0.001).

4.7 EFFECT OF GM-CSF ON THE LPS-INDUCED NEUTROPHIL RESPIRATORY BURST

Tissue macrophages also produce the haematopoetic growth factor GM-CSF when stimulated with LPS. Studies revealed that GM-CSF elicited a neutrophils respiratory burst and that the optimal pretreatment time was 20min, with the optimal concentration of GM-CSF being 100pg/ml (data not

shown). Under these optimal conditions GM-CSF, like TNF-α, significantly enhanced the LPS-induced production of ODRS as measured by lucigenin-dependent chemiluminescence (Fig. 4-10).
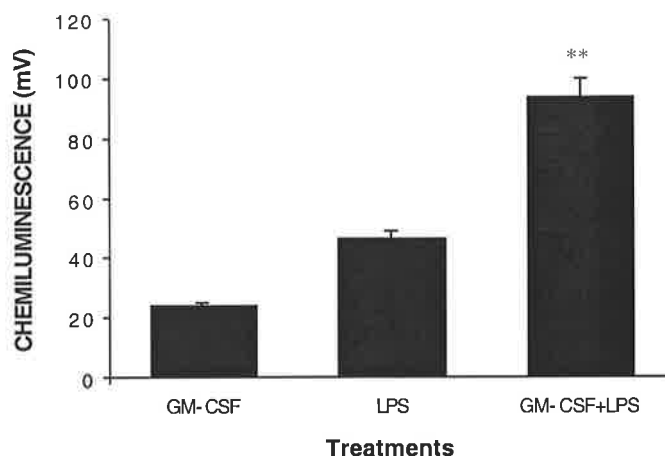
Figure 4-10: Effect of GM-CSF on the LPS-induced neutrophil chemiluminescence. Neutrophils ($1x10^6$ cells) were stimulated with either GM-CSF (100pg/ml, 20min, 37^0C) or HBSS. Then serum was added (1%) and the cells were treated with a second agonist, either LPS (100ng/ml, 50min, 37^0C) or HBSS. The data represent the means ± SEM of the peak value of chemiluminescence (mV) of 4 experiments with neutrophils from different donors.   (** $p < 0.001$ for GM-CSF vs GM-CSF+LPS, LPS vs GM-CSF+LPS and $p < 0.01$ for the sum of GM-CSF and LPS vs GM-CSF+LPS)

4.8   EFFECT OF TNF-α ON THE LPS-INDUCED CHANGES IN THE ADHESIVE PROPERTIES OF NEUTROPHILS

There are two approaches to assess the adhesive properties of neutrophils, one is to assess changes in the level of surface expression of CD11b/CD18, the other is to measure the number of neutrophils attached to surfaces, either serum-coated plastic or endothelial cell layers.

4.8.1   Effect of TNF-α on the LPS-induced adhesion of neutrophils to serum-coated plastic surfaces

The adhesion to serum-coated plastic is an assay directly assessing neutrophil function and was employed first. Neutrophils from 4 different donors were treated in quintuplets and then examined for the degree of adhesion at different incubation periods. Adhesion was measured by the Rose Bengal method as described in Chapter 2. There was an increased adhesion of cells with time following stimulation with TNF-α (20U/ml) or LPS (100ng/ml). The increase was greater when cells were simultaneously treated with both agonists together (Fig. 4-11).
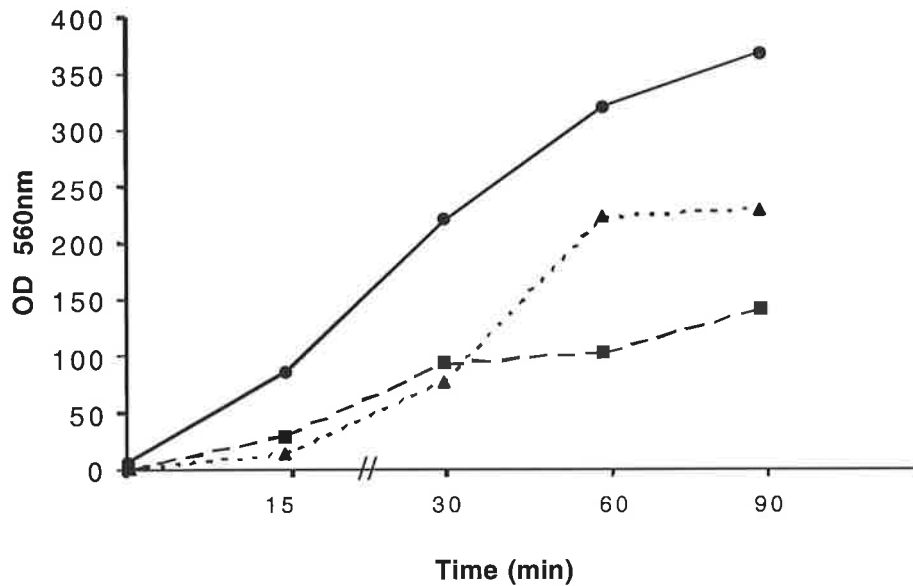
Figure 4-11:   Time characteristics of the adhesion of stimulated neutrophils to plastic. Neutrophils $(1\times10^6$ cells/well) were stimulated with either TNF ($\blacksquare$, 20U), LPS ($\blacktriangle$, 100ng/ml) or TNF+LPS ($\bullet$) for the times indicated. The data are expressed as means of quintuplets of experiments conducted with cells from 4 different donors. The mean optical density (OD) of unstimulated cells was 14 and has been subtracted from the treatments.

When the ability of TNF-α to prime neutrophils for enhanced adhesion in response to LPS was assessed, the optimal TNF-α pretreatment time was 15min and the optimal LPS treatment time was 30min, slightly less than for the effects on the respiratory burst (data not shown). Under these optimal conditions neutrophils $(1\times10^6$ per well) were first pretreated with either TNF-α (20U/well) or diluent (HBSS) and then stimulated with either LPS (100ng/ml) or diluent. Pretreatment with TNF-α significantly enhanced the effects of LPS on adhesion of neutrophils to serum-coated plastic surfaces (Fig. 4-12).
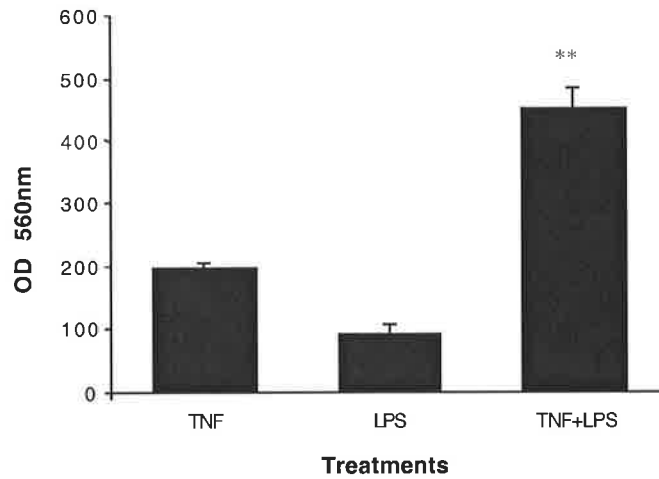
Figure 4-12:   Effect of pretreatment of neutrophils with TNF-α on LPS-induced adhesion to plastic under optimal conditions. Neutrophils (1×10^6 cells/well) were pretreated with TNF-α (20U) or diluent for 15min, then stimulated with either LPS (100ng/ml) or diluent for 30min. The data represent the means ± SEM of sixtuplets of experiments with cells from 5 different donors. The OD of unstimulated cells (17.5) has been subtracted from the treatments.   (** $p < 0.01$)

4.8.2   Effect of TNF-α on the LPS-induced upregulation of CD11b

It has been shown that adhesion of neutrophils to plastic surfaces is associated with the level of expression of CR3 (CD11b/CD18) on the neutrophil surface (Condliffe, 1996). To examine this concept, the influence of TNF-α and LPS on the surface expression of this integrin was studied. TNF-α or LPS caused a concentration-dependent increase in the surface expression of CD11b on neutrophils as assessed by direct flow cytometry. Exppression of this molecule was upregulated in a dose-dependent manner (Fig. 4-13).
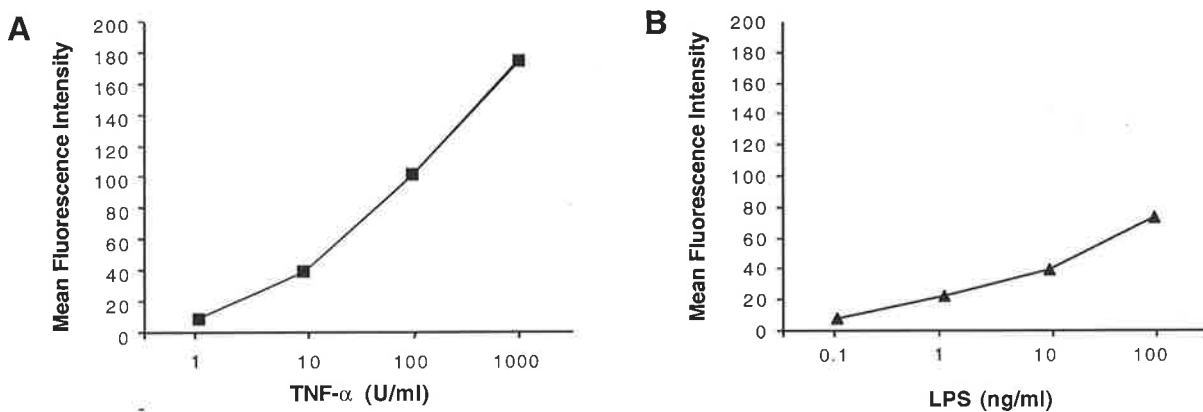
Figure 4-13:   Dose response curves for TNF and LPS in regards to the upregulation of CD11b on neutrophils. **A.** Neutrophils (1×10^6 cells) were incubated for 30min with TNF-α at the concentrations indicated and the expression of CD11b measured by direct flow cytometry. **B.** Cell were stimulated with LPS in the presence of serum (1%) at the concentrations indicated. Both experiments were carried out in duplicates and repeated 3 times with cells from different donors.

To investigate the time-response characteristics of the modulation of CD11b expression the amount of TNF-α and the concentration of LPS were kept constant and the duration of stimulation was varied. With increasing time of incubation the expression of CD11b on the neutrophil surface increased. At 30min, 60min and 90min of treatment the expression of CD11b was significantly higher with TNF+LPS than with either treatment alone (Fig. 4-14).
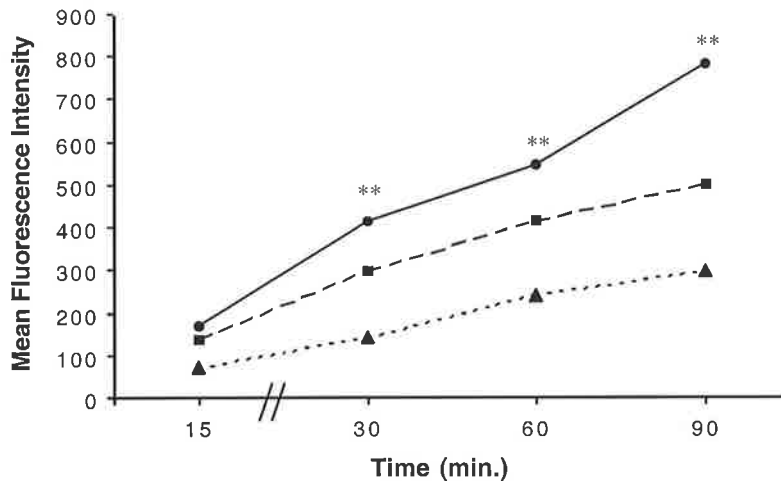
Figure 4-14:   Time characteristics of the upregulation of CD11b on stimulated neutrophils. Neutrophils $(1 \times 10^6/\text{assay})$ were stimulated with either TNF (■, 100U), LPS (▲, 100ng/ml) or TNF+LPS (●) for the indicated times. The data are expressed as means ± SEM of duplicates of experiments with cells from 3 different donors. The mean fluorescence intensity of unstimulated cells was 170 and has been subtracted from the treatments. (** $p < 0.001$ for 30min, 60min and 90min)

Finally, neutrophils were treated under the same conditions as in described in the legend of Fig. 4-12. The upregulation of surface CD11b on neutrophils correlated well with the observed increase in adhesion of neutrophils to plastic (Fig. 4-15).
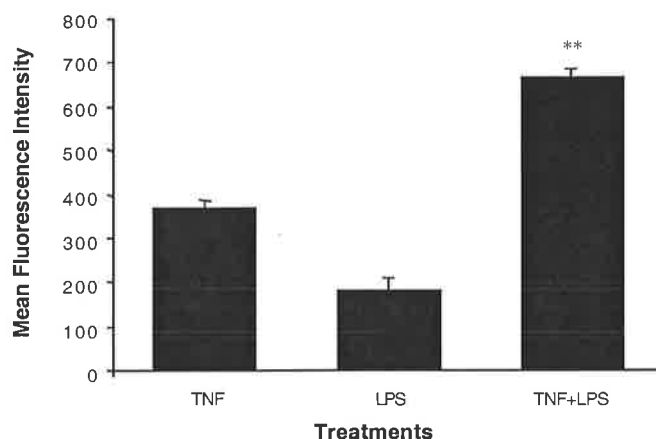
Figure 4-15:   Effect of pretreatment of neutrophils with TNF-α on the LPS-induced upregulation of CD11b. Neutrophils $(1 \times 10^6$ cells/assay) were pretreated with TNF-α (20U) or diluent for 15min, then stimulated with either LPS (100ng/ml) or diluent for 30min. The data represent the means ± SEM of duplicates of 3 independent experiments. The mean fluorescence of untreated cells was 160 and has been subtracted from the treatments. (** $p < 0.001$ for TNF vs TNF+LPS , LPS vs TNF+LPS; $p < 0.01$ for the sum of TNF and LPS vs TNF+LPS)

4.9   SUMMARY AND CONCLUSIONS

The results presented in this chapter show that TNF-α alters neutrophil responses to LPS *in vitro*. Neutrophils pre-exposed to human recombinant TNF-α showed significantly increased oxidative respiratory activity in response to LPS. There was an increase in both, the rate and the total amount of ODRS produced, as measured by lucigenin-dependent chemiluminescence and cytochrome C assay. From dose-response studies it is evident that pretreatment with a wide range of TNF-α concentrations resulted in enhanced sensitivity of neutrophils to LPS. While LPS concentrations in excess of 10ng/ml were required to elicit the neutrophil respiratory burst, pretreatment with TNF-α enabled neutrophils to respond to much lower doses of LPS. For example, a LPS concentration of 1ng/ml led to the same level of ODRS being produced as 100ng/ml of LPS achieved in the absence of pretreatment with the cytokine.

Another key function of the neutrophil, adhesion, was also enhanced by pretreatment with TNF-α, which was associated with the upregulation of the adhesion molecule CD11b on the cell surface. The upregulation of CD11b was rapid and significantly greater in cells treated with TNF-α and LPS compared to neutrophils treated with individual agonists alone.

These results show that LPS and TNF-α are synergistic in the activation of neutrophils which is likely to have significance in the initiation and development of the inflammatory response during bacterial infections. Thus, it is likely that during infection LPS sets up a vicious cycle of neutrophil priming by stimulating TNF-α production by monocytes, which then primes neutrophils to enable them to subsequently respond to LPS in an enhanced manner.

Chapter 5

Upregulation of Vascular Endothelial Cell Adhesion Molecules

5.1   INTRODUCTION

Apart from its barrier function, endothelial cells play an active role in the inflammatory process. Upon activation EC secrete inflammatory mediators, express a pro-coagulatory phenotype and regulate leukocyte traffic by expressing adhesion molecules. A wide range of vascular adhesion molecules has been demonstrated, all of which play an important part in either maintaining tissue structure, wound healing or inflammation (Albelda, 1991). This chapter focuses on the upregulation of the expression of the three luminal endothelial cell adhesion molecules, E-selectin, ICAM-1 and VCAM-1, which are pivotal in the regulation of leukocyte traffic from peripheral blood to tissue-foci of infection or inflammation.

E-selectin (formerly ELAM-1) is not found on the surface of resting EC but is synthesized and expressed within a few hours after cell stimulation. The maximal expression occurs at 5-6 hours and then begins to decline over 24 hours (Bevilacqua et al., 1989). The binding of E-selectin to sialyl Lewis X on leukocytes (CD15) is transient and effects a slowing-down of circulating leukocytes (rolling). Intercellular adhesion molecule-1 (ICAM-1), is constituitively expressed by endothelial cells and is upregulated by stimulation of the cell. Upregulation is observed after 6-9 hours, maximal at 12 hours and persists for greater than 48 hours. The ligands for ICAM-1 on leukocytes are CD11b/CD18 (CR3, Mac-1) and CD11a/CD18 (LFA-1). The interaction between ICAM-1 and these ligands results in the formation of a firm bond between leukocyte and EC (adhesion). The adhesion to the EC leads to migration of the leukocyte through the endothelial cell layer, (diapedesis), following a chemotactic stimulus (Leirisalo-Repo, 1994). Vascular cell adhesion molecule-1 (VCAM-1), like E-selectin is not expressed on resting EC. Its upregulation follows the same time course as ICAM-1. The ligand for VCAM-1 is CD49d/CD29 (VLA-4) on lymphocytes, eosinophils and mononuclear phagocytes (Alon et al., 1995).

Although the individual effects of mediators such as TNF-α and LPS on the expression of the endothelial adhesion molecules E-selectin, ICAM-1 and VCAM-1 are well characterized, little is known about their combined effects on EC. This chapter studies the dose-response relationships and time courses of TNF-$\alpha-$ and LPS-induced effects on the expression of adhesion molecules in HUVEC, and examines whether or not there is synergism between these mediators in this regard.

5.2   THE DOSE-RESPONSE CURVE FOR LPS- AND TNF-α-INDUCED EXPRESSION OF E-SELECTIN

Endothelial cells are known to be sensitive to endotoxin, however, even in systemic infections with gram-negative organisms the concentrations of LPS found in the serum are low, between 0.08-0.3ng/ml (Brandtzaeg et al., 1989; Danner et al., 1991). Thus the dose-response relationship of the LPS-induced expression of E-selectin after 6h was examined. There was no increase in E-selectin

expression with 0.001 and 0.01ng/ml of LPS. With 0.1ng/ml there was statistically significant upregulation of E-selectin, which was almost maximal with 1ng/ml, reaching a plateau with only small additional increases when LPS concentrations were increased to 10, 100 and 1000ng/ml (Fig. 5-1A). An LPS concentration of 0.1ng/ml was chosen for submaximal stimulation in subsequent experiments unless stated otherwise. Similar studies in which the concentration of TNF-α was varied revealed no effect on adhesion molecule upregulation with 0.001 and 0.01U/ml, moderate upregulation with 0.1, 1 and 10U/ml and maximal upregulation with 100, 1000 and 10,000U/ml (Fig. 5-1B). For subsequent experiments suboptimal TNF-α doses of 5U/ml were used.
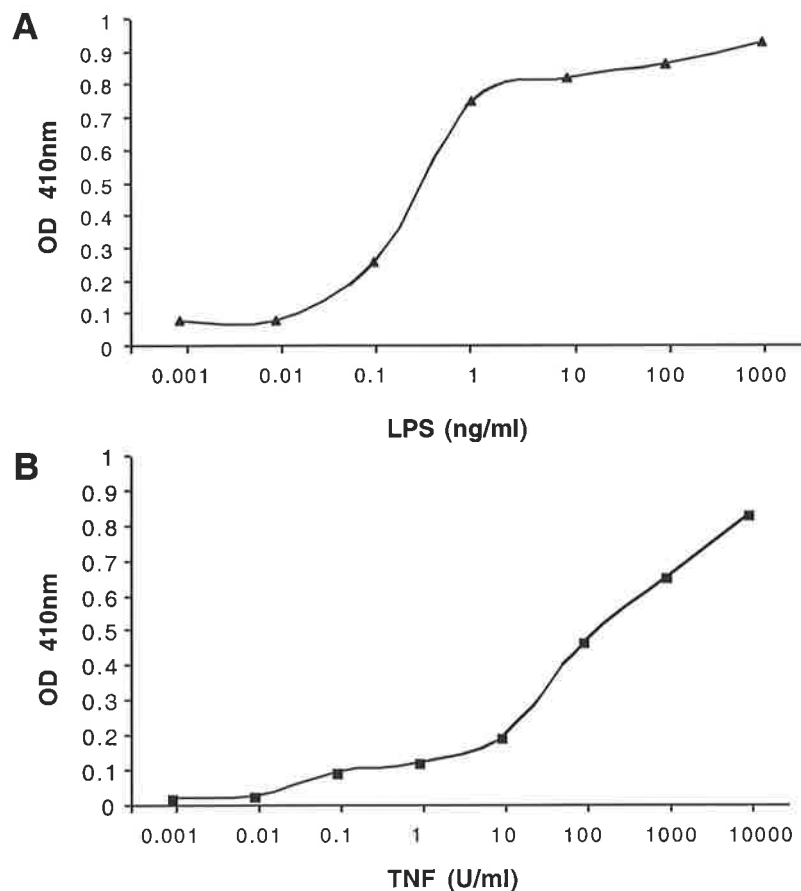
Figure 5-1: The effect of varying the concentration of LPS and TNF-α on the induction of E-selectin expression in endothelial cells. HUVEC (5×10^4 cells/well) were stimulated for 6h with the indicated concentrations of LPS (**A**) or TNF-α (**B**) in the presence of serum (10%). E-selectin was measured by ELISA. The results show the means of 6 determinations from 2 experiments with cells from 2 different cords. The means of untreated cells were 0.007 (**A**) and 0.02 (**B**) and have been subtracted from the LPS and TNF-α treatments.

5.3   TIME COURSE OF THE UPREGULATION OF E-SELECTIN BY TNF-α AND LPS

To determine the time characteristics of the upregulation of E-selectin in our experimental conditions (low doses of agonists, HUVEC grown in 20% human serum) cells were treated with either TNF-α (5U/ml) or LPS (0.1ng/ml). Then sets of cell-containing wells were processed every 0.5h for 6h and

the expression of E-selectin measured by ELISA. E-selectin expression could be detected at 3h, increased further with time and was maximal at 6h (Fig. 5-2). A similar level of expression was found at 8 and 12h (data not shown).
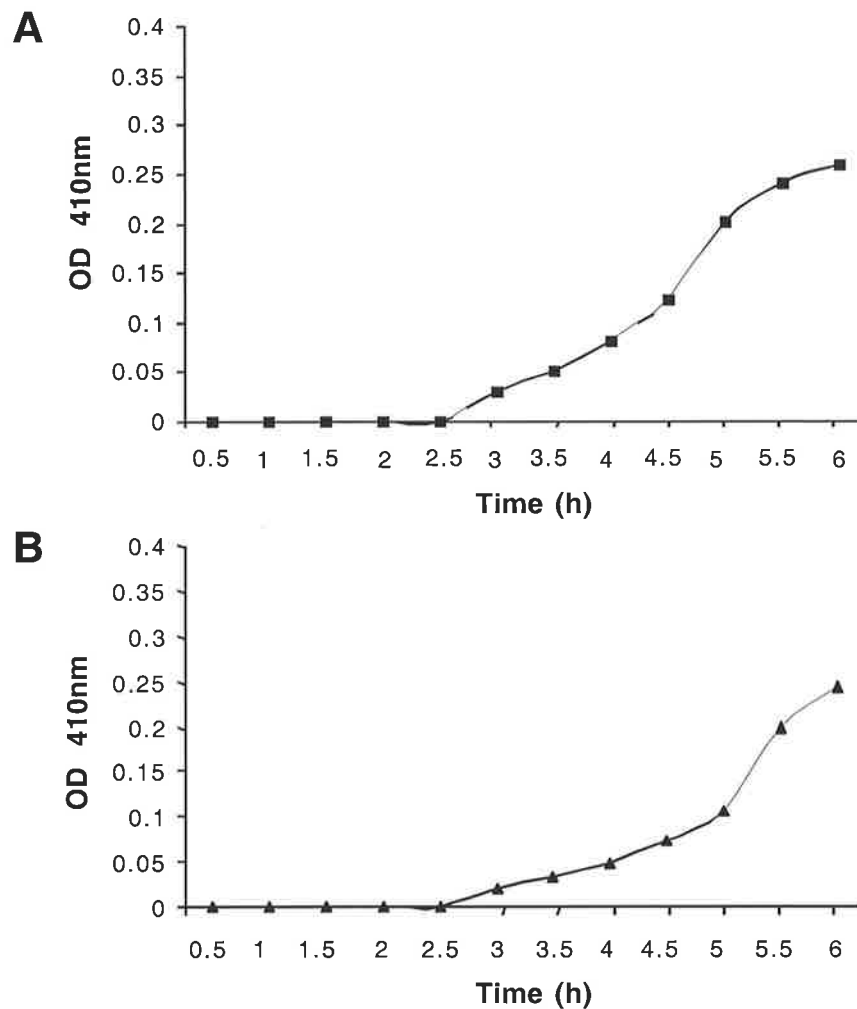
Figure 5-2:   Time course of the induction of E-selectin expression on the surface of endothelial cells by TNF-α and LPS. HUVEC were grown to confluence in 96-well plates (5×10^4 cells/well) and treated with either **A.** TNF-α (5U/ml) or **B.** LPS (0.1ng/ml), for the indicated time periods. E-selectin was measured by ELISA. The results are the means of five determinations from 2 experiments with cells from 2 different cords. The values for unstimulated cells were 0.01 and have been subtracted from the treatments.

5.4   TNF-α AND LPS ACT SYNERGISTICALLY TO UPREGULATE ENDOTHELIAL CELL ADHESION MOLECULES

5.4.1   Adhesion molecules measured by ELISA

HUVEC (5×10^4 cells/well) were grown to confluence in 96-well plates, and cells stimulated with TNF-α or LPS were compared to cells treated with the combined agonists. There was a significantly higher expression of E-selectin with TNF+LPS than with TNF or LPS alone at the 4h and 6h time points.

Furthermore the level of expression achieved by TNF+LPS was greater than the sum of the individual responses for the 6h time point (Fig. 5-3A). Similar responses were seen measuring ICAM-1 and VCAM-1 expression (Fig. 5-4A and Fig. 5-5A).

5.4.2   Adhesion molecules determined by flow cytometry and FACS analysis

While examining endothelial cells in microtiter plates has the advantages of minimal interference and of preserving the intact cell monolayer, the cells are fixed for many hours prior to performing the ELISA assay. This may cause alterations in the cell membrane and originally expressed antigen may be artefactually lost. The synergistic effects of TNF-α and LPS on endothelial adhesion molecule expression were therefore re-examined using an alternative method, flow cytometric analysis. With this assay the cells are only fixed briefly at the end of the staining procedure or not at all (live-cell analysis). The latter technique allows assessments of cell viability at analysis of the data. Potential disadvantages of flow cytometry is the requirement for removing cells from the culture dishes, either mechanically (rubber policeman) or enzymatically (trypsin/EDTA), hence disrupting the physiological monolayer environment. In the following experiments trypsin/EDTA was used at low concentrations and for the shortest time possible by watching detachment of cells from the floor of the culture dish. Cells were placed on ice immediately after harvesting and the trypsin removed by washing. E-selectin was measured at 2,3,4 and 6 hours.

There was significantly greater upregulation of E-selectin with TNF+LPS as opposed to the effect of treatment with the individual stimuli. The effect of co-stimulation was also significantly greater than the sum of individual responses at 3,4 and 6 hours (Fig. 5-3B). ICAM-1 and VCAM-1 were measured at 5.5, 7.5, 9.5, and 11.5h. Again, the results were similar to those obtained with ELISA (Fig. 5-4B and Fig. 5-5B).
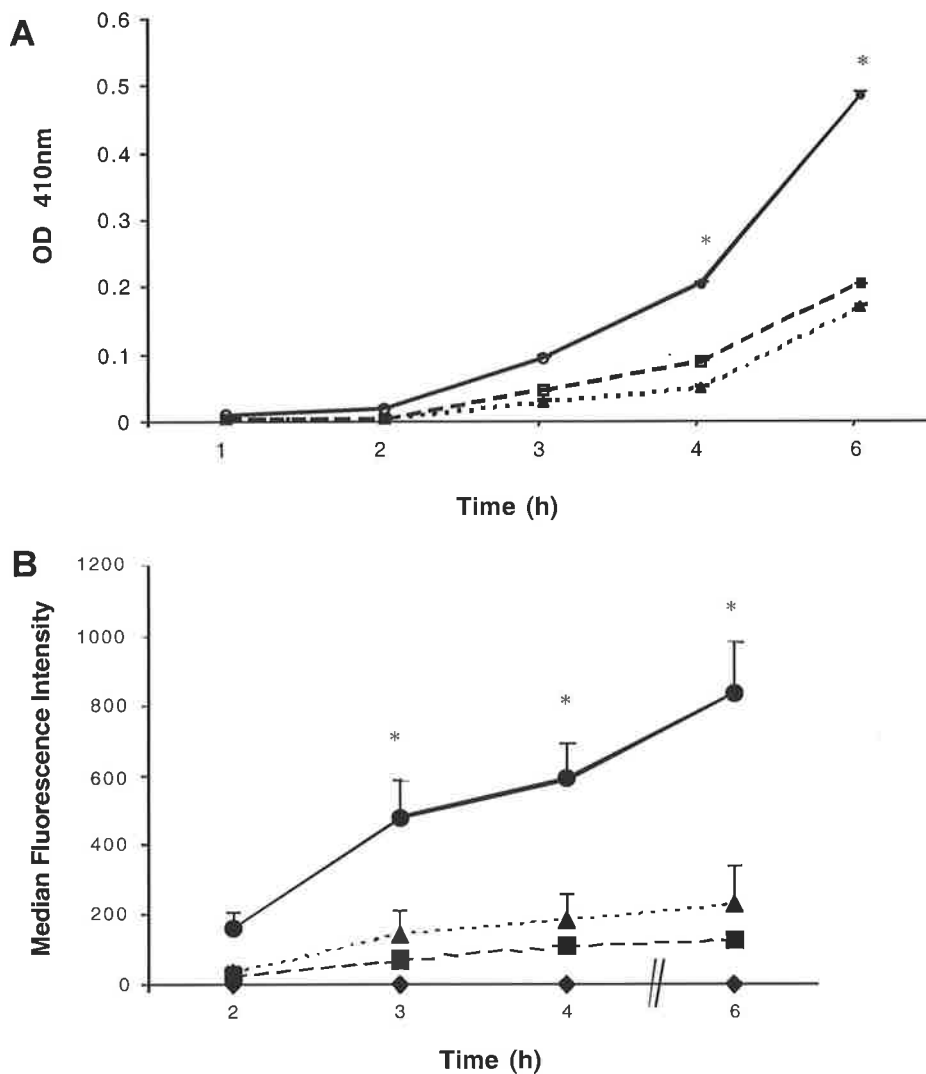
Figure 5-3: Effect of combined TNF-α and LPS treatment on E-selectin expression by endothelial cells. HUVEC (5×10^4 cells/well) were treated with either TNF (■, 5U/well) or LPS (▲, 0.1ng/ml) or TNF+LPS (●) for the times indicated. **A.** E-selectin was measured by ELISA. The value for untreated cells was 0.009 and was subtracted from the treatments. The results are the means ± SEM of 5 experiments with cells from 5 cords, each run in triplicate. (* TNF vs TNF+LPS, LPS vs TNF+LPS p < 0.001, sum of TNF and LPS VS TNF+LPS p<0.01). **B.** E-selectin was measured by flow cytometry. Cells were treated with diluent (◆), TNF ($5U/10^5$ cells ■), LPS (0.1ng/ml ▲) or TNF+LPS (●) for the times indicated. The mean fluorescence intensity of 10,000 cells per sample was determined. The data represent the means ± SEM of 5 experiments, each with cells from a different cord. (* TNF or LPS vs TNF+LPS, p<0.001 at 3, 4, and 6h. Sum of TNF and LPS vs co-stimulation (TNF+LPS) p< 0.01 at 3, 4, and 6h.)
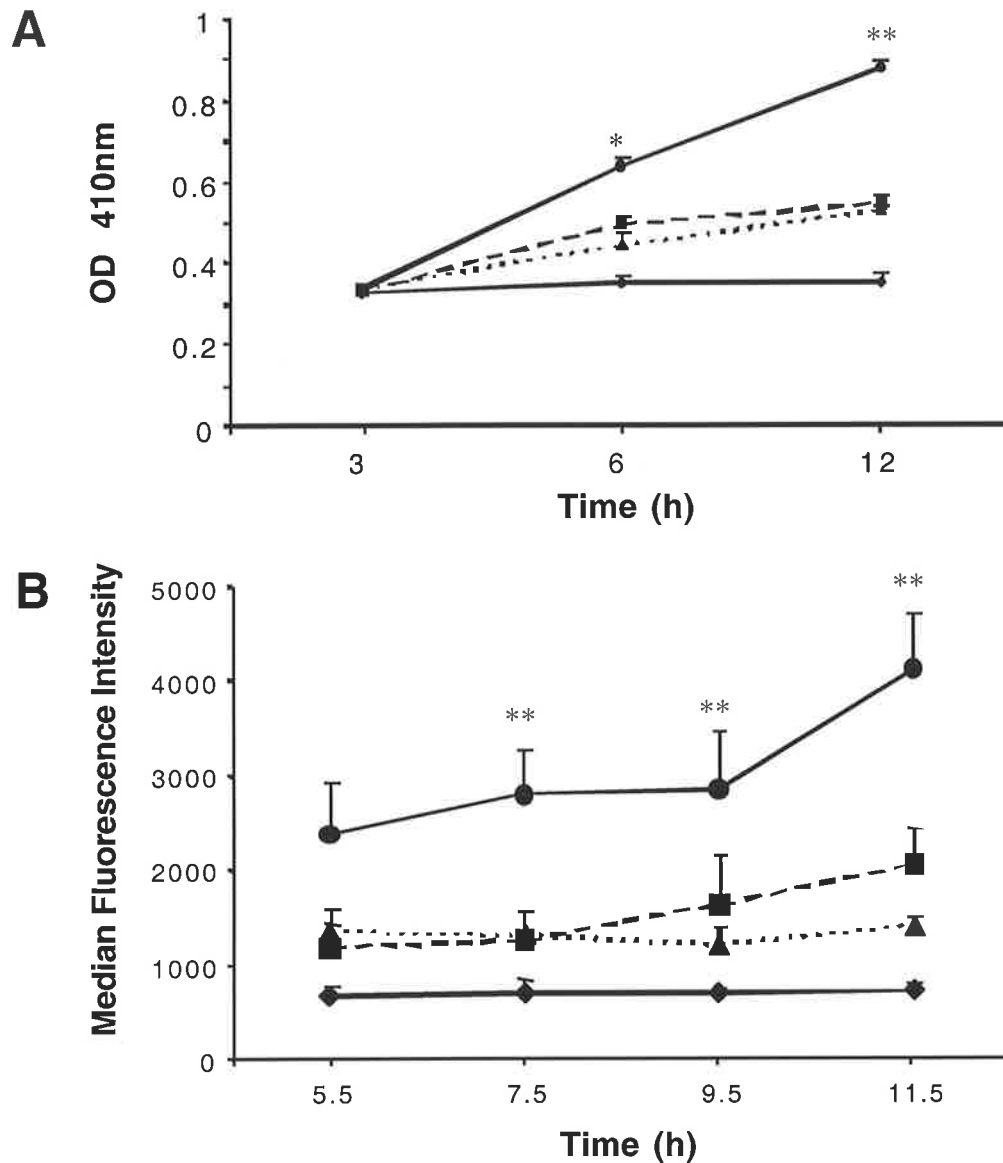
Figure 5-4:   Effect of combined TNF-α and LPS treatment on ICAM-1 expression by endothelial cells. Treatment conditions were as outlined in the legend of Fig. 5-3. Both graphs show the means ± SEM of 4 experiments each conducted with cells from 4 different cords and in triplicate. Key: cells treated with diluent only (◆), TNF (■), LPS (▲) and TNF+LPS (●) **A.** The expression of ICAM-1 for cells treated for the times indicated was measured by ELISA. (* $p < 0.01$ for TNF vs TNF+LPS, LPS vs TNF+LPS, ** $p < 0.001$ for TNF vs TNF+LPS, LPS vs TNF+LPS, $p < 0.01$ for sum of TNF and LPS vs TNF+LPS) **B.** The expression of ICAM-1 measured by flow-cytometry. ($p > 0.05$ at 5.5h, $p < 0.001$ at 7.5h, $p < 0.01$ at 9.5h, and $p < 0.001$ at 11.5h. Sum of TNF and LPS vs co-stimulation (TNF+LPS): $p < 0.01$ at 7.5h, 9.5h, and 11.5h)
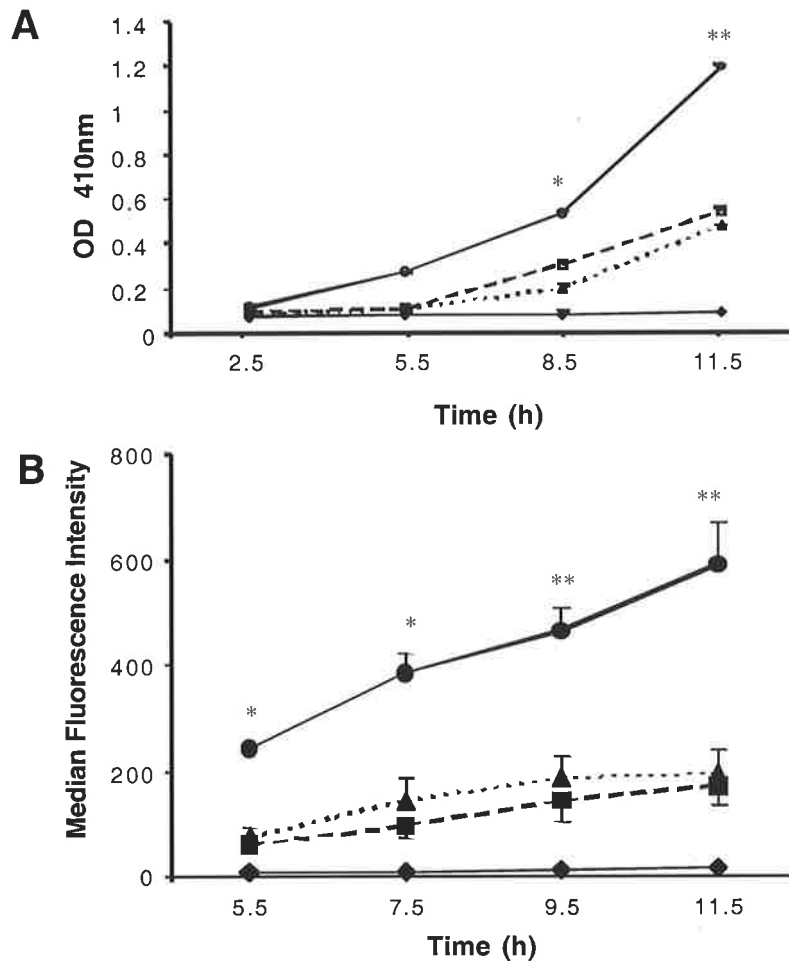
Figure 5-5:   Effect of combined TNF-α and LPS treatment on VCAM-1 expression by endothelial cells. Treatment conditions were as outlined in the legend of Fig. 5-3. The data presented are the means ± SEM of 4 experiments each conducted with cells from 4 different cords and each in triplicate. Key: cells treated with diluent only (◆), TNF (■), LPS (▲) and TNF+LPS (●) **A.** The expression of VCAM-1 in cells treated for the times indicated was measured by ELISA. (* $p < 0.01$ for TNF vs TNF+LPS, LPS vs TNF+LPS, ** $p < 0.001$ for TNF vs TNF+LPS, LPS vs TNF+LPS, $p < 0.01$ for sum of TNF and LPS vs TNF+LPS, ANOVA.) **B.** The expression of VCAM-1 was measured by Flow-cytometry. (TNF or LPS vs TNF and LPS $p < 0.01$ at 5.5h, 7.5h, and 9.5h, $p < 0.001$ at 11.5h. Sum of TNF and LPS vs co-stimulation (TNF+LPS) $p < 0.05$ at 9.5h, and $p < 0.01$ at 11.5h)

5.5   SUMMARY AND CONCLUSIONS

TNF-α and LPS at suboptimal concentrations, which induced no alteration in expression of endothelial cell adhesion molecules, caused significantly greater expression of E-selectin, ICAM-1 and VCAM-1 when added together. This increase in expression was significantly greater than the sum of the responses achieved by TNF-α or LPS individually. Similar results were obtained when the expression of endothelial cell adhesion molecules was measured with ELISA and flow cytometry.

Chapter 6

**Effects on ICAM-1 Expression
in Respiratory Epithelial Cells**

6.1   INTRODUCTION

The lower respiratory tract is the largest epithelial surface exposed to the external environment and therefore the respiratory epithelium constitutes an important barrier against infection by inhaled pathogens (Toews, 1986). In addition to its barrier function the pulmonary epithelium contributes to the immune and inflammatory response by antigen processing, antigen presentation, and by releasing mediators which modify responses of inflammatory cells (Jordana et al., 1992; Smart et al., 1994). When respiratory epithelial cells are stimulated these cells produce a wide range of mediators which act on immune cells (Table 6-1).

Table 6-1:   Mediators produced by respiratory epithelial cells

Mediator	Reference
Cytokines and growth factors	
MCP-1	Erger et al., 1995
RANTES	Stellato et al., 1995
IL-11	Elias et al., 1994
IL-3, IL-6, IL-8	Nakamurra et al., 1991
IFN-γ	Hahon et al., 1989
TNF-α	Khair et al., 1994
GM-CSF	Newton et al., 1995
TGF-β	Khalil et al., 1994
PDGF	Sariban et al., 1988
Complement components	
C2,C3,C4	Khirkwadkar et al., 1993
Antibiotic peptides	
Tracheal anti-microbial peptide (TAP)	Diamond et al., 1996
Other immune-modulators	
Surfactant	Simon et al., 1995
Proteinase inhibitors	Sallenave et al., 1993
H_2O_2, MnSOD mRNA	Lopez et al., 1988
Urokinase	Berry et al., 1991
Fibronectin	Berry et al., 1991
Collagen type IV	Berry et al., 1991
Tissue factor (TF)	Berry et al., 1991
Thrombospondin	Berry et al., 1991
PAI-1 and other anticoagulants	Berry et al., 1991

In addition, stimulated respiratory epithelial cells show an increase in expression of the adhesion molecule ICAM-1, which is likely to influence the movement of inflammatory leukocytes. This stimulation of the epithelium may be brought about either by bacteria directly interacting with the

epithelial surface molecules such as fibronectin (Pottratz et al., 1991) and vitronectin (Rubens et al., 1992) or cell-activating products released by bacteria. Bacteria may finally invade epithelial cells (Chi et al., 1991; Schipper et al., 1994; Talbot et al., 1996).

However, we still understand very little of the regulation of respiratory epithelial cells by mediators released by either tissue cells or microorganisms interacting with epithelium. Epithelial cells are likely to be influenced by a milieu of mediators and we have addressed this with respect to TNF-α, LPS, IL-1β and IFN-γ and also LPS.

6.2   STIMULATION OF ICAM-1 ON RESPIRATORY EPITHELIAL CELLS BY TNF-α, IL-1β AND IFN-γ

Although primary isolates and cultures of human alveolar type II cells have been described using post-mortem explants from heart-lung transplantation patients (Dobbs, 1990), such material is not widely available. There are at least 14 different human immortalized cell lines commercially registered. Of these, A549 has been used most widely and is an accepted model to study respiratory epithelial function. (Smith, 1977; Nardone et al., 1979). This immortalized cell line was originally obtained from an adeno-carcinoma of human lung (Lieber et al., 1976). The cells have retained most of the features of pulmonary alveolar type II pneumocytes. A549 cells produce IL-8 in response to TNF-α and IL-1β (Smart et al., 1994).

ICAM-1 is constituively expressed by nasal, tracheal and alveolar type I epithelial cells, but it is not found on unstimulated type II pneumocytes (Simon et al., 1995). Agents such as TNF-α, IL-1β and IFN-γ upregulate ICAM-1 mRNA in type II pneumocytes and induce the expression of ICAM-1 on the cell surface. ICAM-1 can be detected at 4h and reaches a maximal expression at 24 to 48h after stimulation. Like primary type II cells, resting A549 cells express very little ICAM-1, but the expression of this molecule can be induced by TNF-α, Il-1β and IFN-γ (Altman et al., 1993; Mickelson et al., 1995). ICAM-1 serves to enhance the adhesion of neutrophils and monocytes to epithelial cells (Tosi et al., 1992). It has been found to be upregulated in asthma, where it is thought to mediate increased eosinophil adhesion to airway epithelium (Wegner et al., 1990). ICAM-1 also promotes cell invasion and infection by viruses such as rhinovirus and respiratory syncytial virus (RSV) by facilitating attachment (Chini et al., 1998; Turner et al., 1999). In the experiments described below the expression of ICAM-1 was used as a marker for epithelial cell activation. The level of expression of this molecule on the cell surface was determined by flow cytometry and FACS analysis.

In investigations to examine the effects of cytokines on ICAM-1 expression on epithelial cells, A549 cells (1×10^5/assay) were treated with a wide range of concentrations of TNF-α, IL-1β and IFN-γ. The results showed that the expression of ICAM-1 was significantly increased when cells were treated with $\geq$10U/ml of TNF-α, $\geq$0.1ng/ml of IL-1β and $\geq$1000U/ml of IFN-γ. The increase was

cytokine concentration-dependent. There was a continuous increase in upregulation with TNF-α, while IL-1β-induced upregulation reached a plateau with 1ng/ml. The response to IFN-γ was only modest, 10,000U/ml produced only a 5-fold increase over baseline (Fig. 6-1).

Treatments
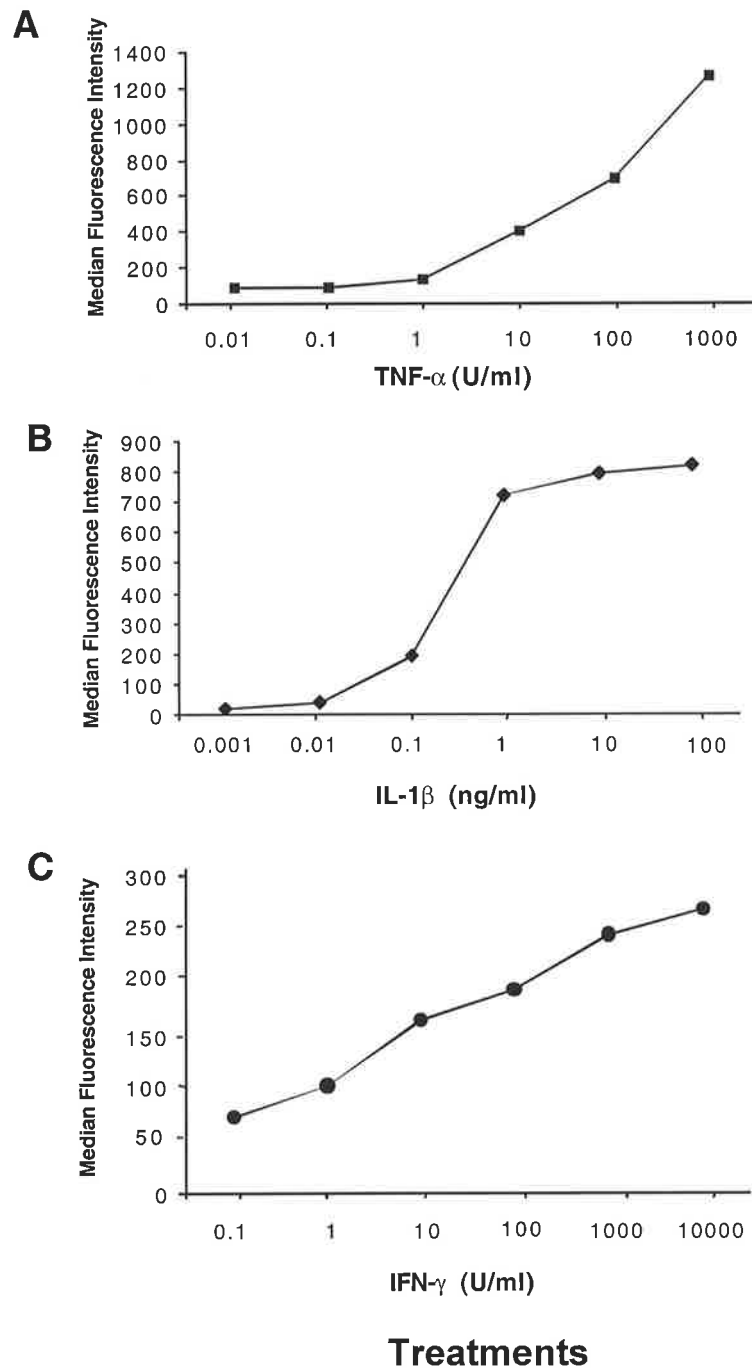
Figure 6-1:   Effects of TNF-α, IL-1β and IFN-γ on the expression of ICAM-1 on the surface of respiratory epithelial cells. A549 cells were treated with either TNF-α (**A**), IL-1β (**B**) or IFN-γ (**C**) for 24h with the cytokine concentrations as indicated. The results shown are the means of 3 experiments conducted in duplicate. The results for untreated cells were 65, 51 and 48 respectively and have been subtracted from the treatments. ICAM-1 was measured by indirect flow cytometry and FACS analysis, 10,000 viable cells per sample were analyzed (as assessed by 7-AAD staining).

6.3   SYNERGISM BETWEEN CYTOKINES

To evaluate whether cytokines operate in concert to activate pulmonary epithelial cells, A549 cells ($1x10^5$/assay) were treated with suboptimal concentrations of TNF-α (10U/ml), IL-1β (0.1ng/ml) and IFN-γ (1000U/ml) for 24h. Additionally, cells received combinations of treatments, TNF+IL-1, TNF+IFN-γ, IL-1+IFN-γ as well as with all three cytokines together. While TNF-α and IL-1β were additive, there was synergism in ICAM-1 upregulation when IFN-γ was combined with each of the other cytokines. The response was moderate for IL-1+IFN-γ and marked for TNF+IFN-γ. Adding all three cytokines together did not achieve a greater increase than TNF+IFN-γ (Fig. 6-2).
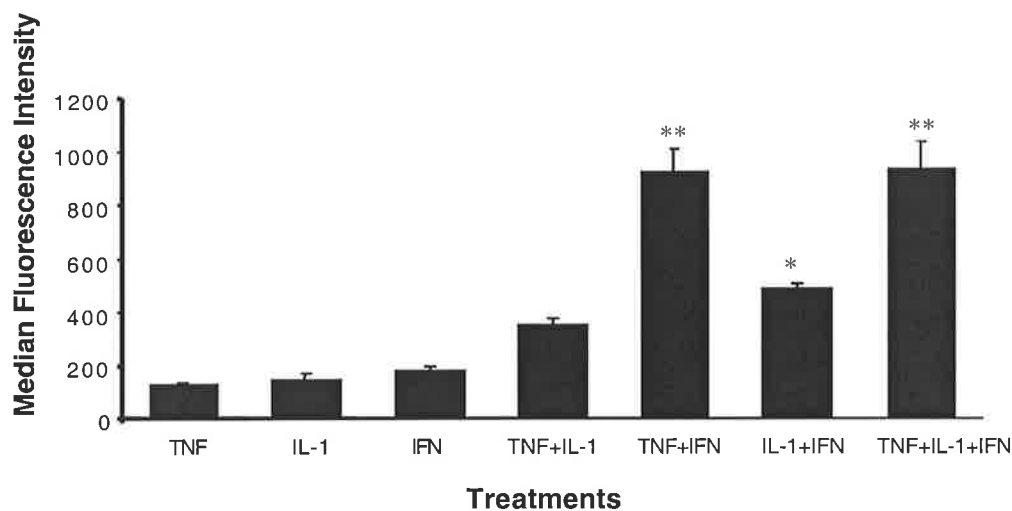
Figure 6-2:   Effect of combinations of different cytokines on the upregulation of ICAM-1 on A549 cells. Cells ($1x10^5$/assay) were treated for 24h with either TNF-α (10U/ml), IL-1β (0.1ng/ml), IFN-γ (1000U/ml) or with combinations of these agonists as indicated. The data represent the means ± SEM of 2 experiments, each conducted in duplicates. The value for untreated cells was 65 and has been subtracted from the treatments. Synergism is defined as the effect of the combined treatment being significantly greater than the sum of the effects of individual agonists. (* $p < 0.01$ for IL-1+IFN vs TNF or IL-1 or IFN; ** $p < 0.001$ for TNF+IFN or TNF+IL-1+IFN vs TNF or IL-1 or IFN, alone or combined, or TNF+IL-1 or IL-1+IFN; n.s. for TNF+IFN vs TNF+IL-1+IFN)   [**TNF**, TNF-α; **IL-1**, IL-1β, **IFN**, IFN-γ]

6.4   ENHANCEMENT OF THE EFFECT OF CYTOKINES BY LPS

Airway epithelium in general, and in particular type II pneumocytes are known to be insensitive to LPS. To confirm this A549 cells ($1x10^5$/assay) were treated with a range of concentrations of LPS (*E. coli* K-235) for 24h. Even with concentrations of LPS as high as 10μg/ml there was no significant upregulation of ICAM-1 as compared to baseline (Fig. 6-3).
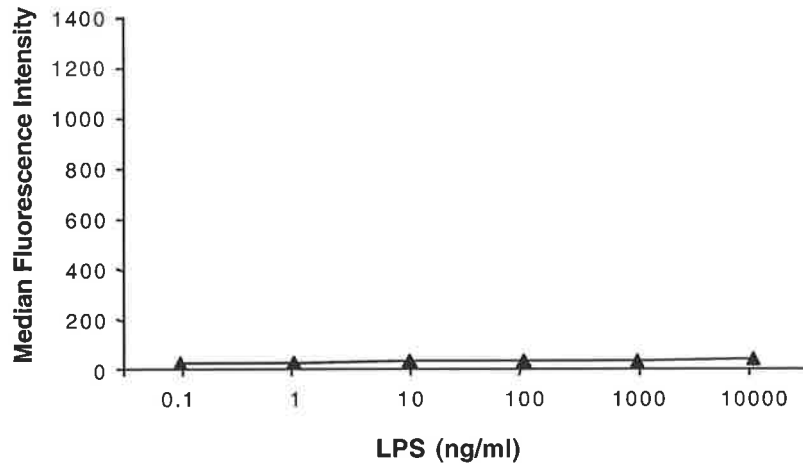
Figure 6-3:   Effect of LPS on the expression of ICAM-1 on A549 cells. Epithelial cells (1×10^5/well) were treated with increasing concentrations of LPS. The results are the means of duplicates of 2 independent experiments. The value for untreated control cells was 52 and has been subtracted from all treatments. None of the values was significantly greater than the baseline.

Previous studies have shown that LPS in conjunction with TNF-α, IL-1β and IFN-γ caused an increase in inducible nitric oxide synthetase (iNOS) in A549 cells (Watkins et al., 1997). Accordingly, A549 cells (1×10^5/assay) were treated for 24h with either single agonists, as in section **6.5** above, or LPS (10μg/ml) or all 3 cytokines with and without the addition of LPS. The addition of LPS to the cocktail of the 3 cytokines produced more than a doubling of ICAM-1 upregulation (Fig. 6-4). Similar effects were seen when only 1μg/ml of LPS was used (data not shown).
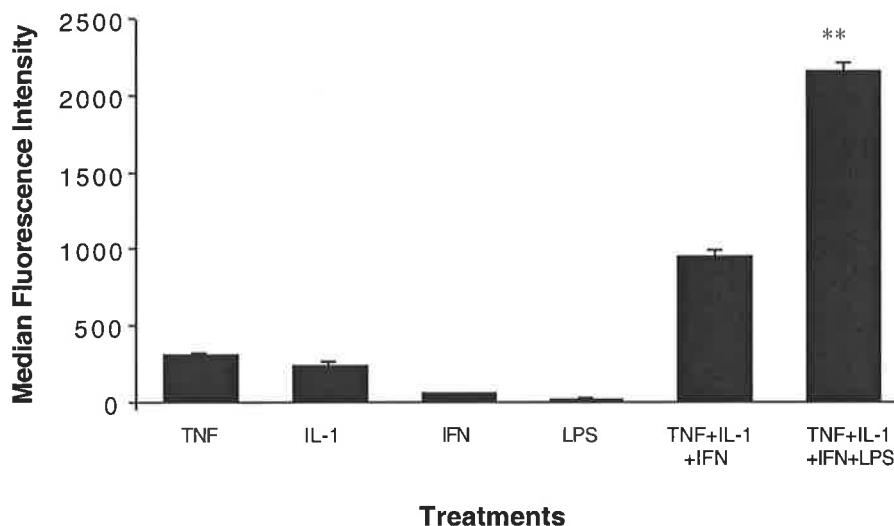
Figure 6-4:   Synergistic effects of LPS and cytokines on the expression of ICAM-1 in respiratory epithelial cells. A549 cells (1×10^5/assay) were treated with either TNF-α (10U/ml), IL-1β (100pg/ml), IFN-γ (1000U/ml), LPS (10μg/ml), or the indicated combinations of agonists for 24h. ICAM-1 expression was determined by FACS analysis. Each value represents the mean of 2 independent experiments conducted in duplicates. The value for untreated cells was 11 and has been subtracted from the treatments. (** p < 0.001 for TNF+IL-1β+IFN-γ+LPS vs TNF+IL-1β+IFN-g)

It was of interest to examine the effect of the addition of LPS on the upregulation of ICAM-1 by each individual cytokine. LPS (1μg/ml) enhanced the effect of IFN-γ (1000U/ml), but had no effect on the TNF-α (10U/ml) and IL-1β (0.1ng/ml)-induced expression of ICAM-1 on A549 cells (Fig. 6-5).
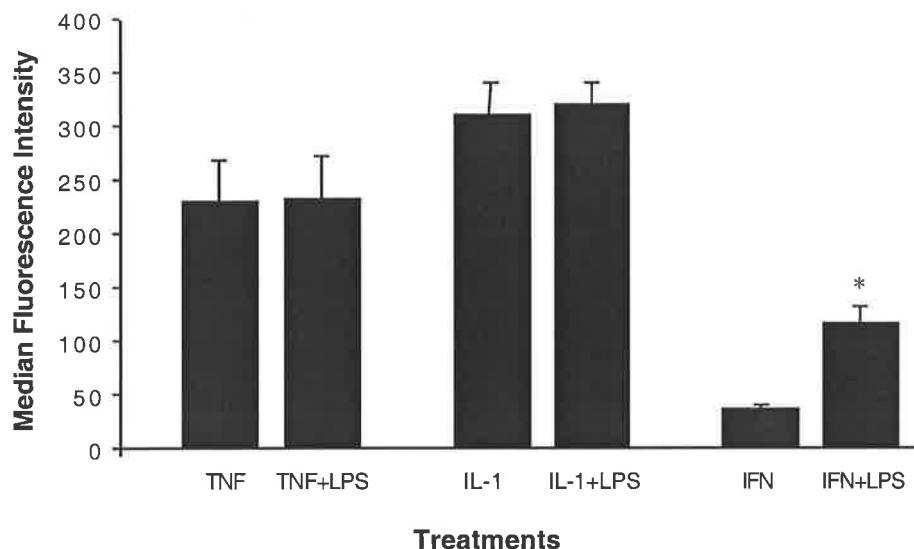
Figure 6-5:   Effect of LPS on cytokine-induced upregulation of ICAM-1 on respiratory epithelial cells. A549 cells (1x10^5/assay) were treated for 24 hours with either TNF-α (10U/ml), IL-1β (0.1ng/ml), IFN-γ (1000U/ml) with or without LPS (1μg/ml). ICAM-1 expression was determined by indirect flow cytometry and FACS analysis. The data represent the means of duplicates from 2 different experiments. The value for LPS alone was 19 and has been subtracted from all treatments. (* p < 0.01 for IFN-γ vs IFN-γ+LPS; ns for TNF vs TNF+LPS and for IL-1β vs IL-1β+LPS)

In these experiments LPS and the cytokines were added together. We then examined whether pre-tereatment of pulmonary epithelial cells with cytokines rendered them susceptible to LPS, A549 cells (1x10^5/assay) were pretreated with TNF-α (10U/ml), IL-1β (0.1ng/ml) or IFN-γ (1000U/ml) for 0,1,2,3 or 4h. After a further 24h of incubation there was no significant increase in the TNF-α and IL-1β-induced responses, while pretreatment with IFN-γ was associated with a significantly greater surface expression of ICAM-1 by LPS. This increase was maximal at 2h of preincubation (Fig. 6-6).
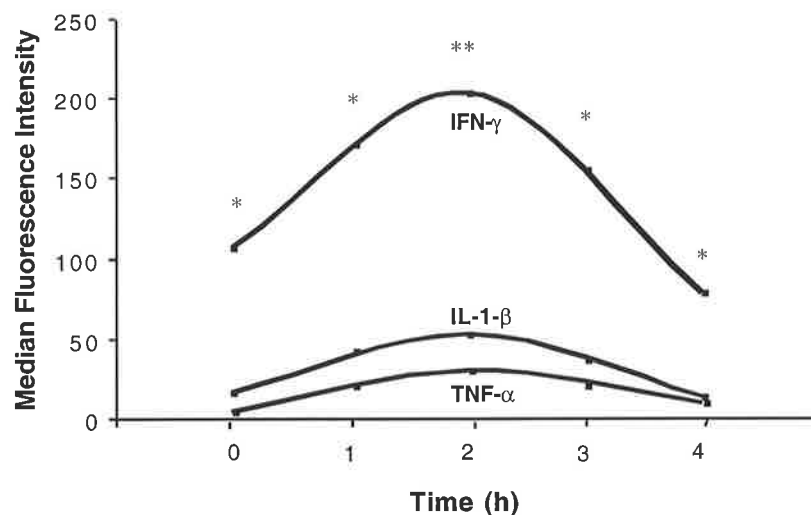
Figure 6-6:   LPS-enhanced cytokine-induced ICAM-1 expression on A549 cells in relationship to pretreatment-times with various agonists. A549 cells (1×10^5/well) were incubated with either TNF-α (10U/ml), IL-1β (0.1ng/ml) or IFN-γ (1000U/ml) in the presence or absence of LPS (1μg/ml) for the times indicated. The data are expressed as means of the LPS co-treated wells with the means of the agonist-only wells subtracted. The data are derived from 2 experiments, each conducted in duplicates. The value for cells treated with LPS alone was 18. (* $p < 0.01$, ** $p < 0.001$)

6.5   SUMMARY AND CONCLUSIONS

The data demonstrate that the cytokines TNF-α and IL-1β are able to activate pulmonary epithelial cells as assessed by the upregulation of the adhesion molecule ICAM-1 on the cell surface. The effect of these cytokines could be significantly enhanced with IFN-γ, which had only modest effects on ICAM-1 expression when used alone.

While bacterial lipopolysaccharide (LPS) had no effect on ICAM-1 expression in A549 cells, it was able to significantly increase the expression of this integrin molecule in combination with a cocktail of cytokines (TNF-α, IL-1β and IFN-γ). When these cytokines were tested as to whether they could individually alter the susceptibility of A549 cells to LPS, only IFN-γ exerted this effect. This is consistent with the observation by others that LPS can not induce IL-6 mRNA in alveolar type II cells, yet it enhances IFN-γ-induced production of IL-6 mRNA (Crestani et al., 1994).

Chapter 7

The Effect of TNF-α and LPS on Cell Signalling in Human Umbilical Vein Endothelial Cells

7.1   INTRODUCTION

The synergistic properties of TNF-α and LPS could be operating at a number of different levels to culminate in an increased expression of adhesion molecules. This includes intracellular signalling, as well as transcription. TNF-α and LPS each individually are known to activate the transcription factors NF-κB through IκB degradation, or ATF-2 and Elk-1 through the MAP kinases p38, JNK and ERK (see section 1.6 for details). As described in Chapter 5 TNF-α and LPS were synergistic in upregulating the expression of the endothelial cell adhesion molecules E-selectin, ICAM-1 and VCAM-1. This occurred with suboptimal concentrations of agonists that were insufficient to induce upregulation of adhesion molecules if those mediators were used alone. While there are extensive studies defining the involvement of signalling molecules in the activation of cells by individual stimuli it is not clear how different mediators affect these signalling cascades when more than one agonist receptor is engaged. We have examined this with respect to the activation of human umbilical vein endothelial cells by TNF-α and LPS.

7.2   SYNERGISM AT THE LEVEL OF mRNA

To determine whether or not the combined effect of TNF-α and LPS on adhesion molecule expression was also reflected in an increase in adhesion molecule mRNA production HUVEC were treated with TNF (25U/ml) and LPS (0.1ng/ml), doses which produced synergistic responses (Chapter 5). The amount of E-selectin mRNA was measured after 2h. The results showed that treatment with TNF-α and LPS resulted in a significantly greater amount of E-selectin mRNA compared to each agonist alone, and this increase was also greater than the sum of the responses caused by TNF-α and LPS alone (Fig. 7-1).
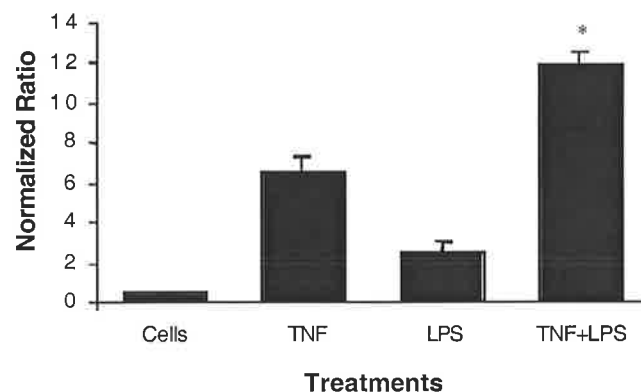
Figure 7-1:   The effects of combined treatment with TNF-α and LPS on E-selectin mRNA. E-selectin mRNA was assayed as described in methods. HUVEC monolayers were treated with either diluent (Cells), TNF-α (25U/ml), LPS (0.1ng/ml) or TNF + LPS for 2h. The columns represent the means ± SEM of triplicates and are expressed as normalized ratio-values (as described in methods) of two experiments each conducted with cells from separate cords.   TNF vs TNF+LPS: $p < 0.01$; LPS vs TNF+LPS: $p < 0.001$; sum of TNF and LPS vs co-stimulation (TNF+LPS): $p < 0.01$.

7.3   EFFECTS ON TRANSCRIPTION FACTOR NF-κB

Both TNF-α and LPS are known to activate NF-κB (section 1.6.4). This activation is a transient phenomenon which peaks between 1-4h after a stimulus and diminishes thereafter, coinciding with the reconstitution of IκB (Read et al., 1994). Endothelial cells were treated with diluent, TNF (25U/ml), LPS (0.1ng/ml) or TNF-α and LPS for 2.5h. Nuclear extracts were prepared and NF-κB (p65) was assayed by Western Blot (described in Methods). A representative scan of an image obtained with enhanced chemiluminescence is shown (Fig. 7-2A). The amount of NF-κB that had accumulated in the nucleus after the combined treatment with TNF-α and LPS was significantly greater than that obtained with either TNF-α or LPS alone, as well as greater than the sum of these individual responses (Fig. 7-2B).
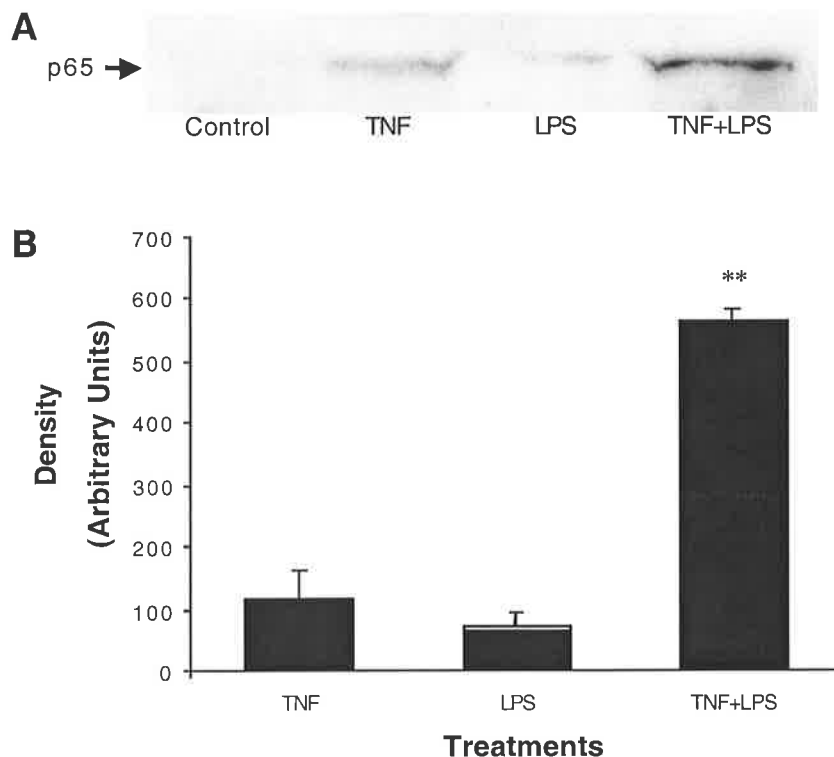
Figure 7-2:   Effect of TNF-α and LPS on NF-κB (p65). The amount of NF-κB that was present in the nuclear extracts of either untreated endothelial cells (2.5x10^6) or cells treated with TNF-α (25U/ml), LPS (0.1ng/ml) or TNF+LPS for 2.5h, was measured by Western Blot. The densities of the bands were quantified by an Image Quant™ scanner and Image Quant software (version 3.3) and are expressed as " Arbitrary units". **A.** Representative bands obtained with enhanced chemiluminescence are shown. **B.** The data are expressed as means ± SEM derived from 4 experiments, each conducted with cells from a different cord. The mean of the values of cells treated with diluent (38 arbitrary Units) has been subtracted from all other treatments.   * p < 0.001 for sum of TNF-α and LPS vs co-stimulation (TNF+LPS).

When nuclear extracts of HUVEC (treatment conditions as above) were assayed after 2.5, 8, 20 and 28h of incubation in the presence of both TNF-α and LPS, there was still an increase in NF-κB after 28h compared to either agonist alone (Fig. 7-3).
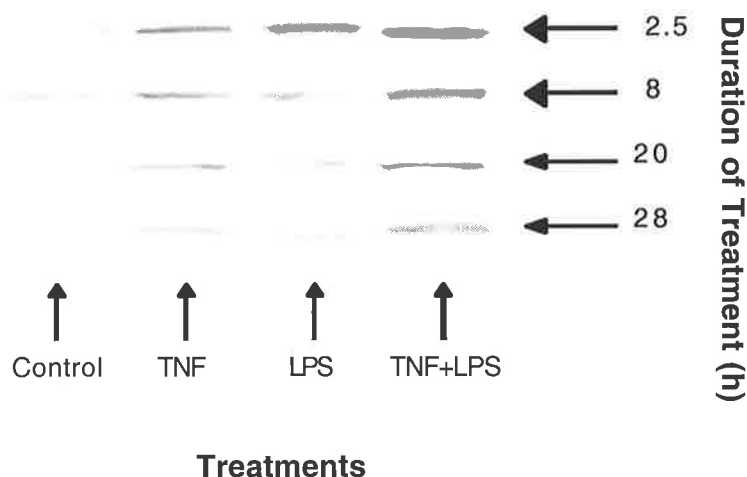
Figure 7-3:   Time course of NF-κB (p65) activation in HUVEC. Cells were harvested and processed at 2.5, 8, 20 and 28h. The amount of NF-κB (p65) in nuclear extracts were measured by Western Blotting. The images of the bands were obtained as described in the legend of Figure 7-2.

7.4   EFFECTS OF TNF-α AND LPS ON MAP KINASES

Previous studies showed that maximal activation of the MAP kinases ERK, JNK and p38 occurs 10-15min after the stimulation of endothelial cells with either TNF-α or LPS (Read et al., 1997). Synergism between TNF-α and LPS was also examined with respect to these kinases. HUVEC (2.5×10^6 cells/assay) were treated with diluent, TNF-α (25U/ml), LPS (0.1ng/ml) or TNF+LPS and the activities of MAP kinases were determined as described in Methods.

7.4.1   ERK

The activity of ERK was determined by measuring the ^{32}P phosphorylation of myelin basic protein and resolving the samples by SDS-PAGE (see Methods). A representative scan obtained with an Instant Imager™ is shown (Fig. 7-4A). The results showed that ERK activity was constituitively expressed. While this activity was increased by treatment with either TNF-α or LPS (Fig. 7-4B), these mediators had no synergistic effect on ERK activity, even when the concentrations of TNF-α and LPS were increased 100-fold (not shown). These findings are in agreement with other studies, which demonstrate that the ERK cascade does not play a major role in endothelial cell activation by either TNF-α or LPS (Read et al., 1997).

A

Phosphorylated ⟶
myelin basic protein

Control   TNF   LPS   TNF+LPS

B
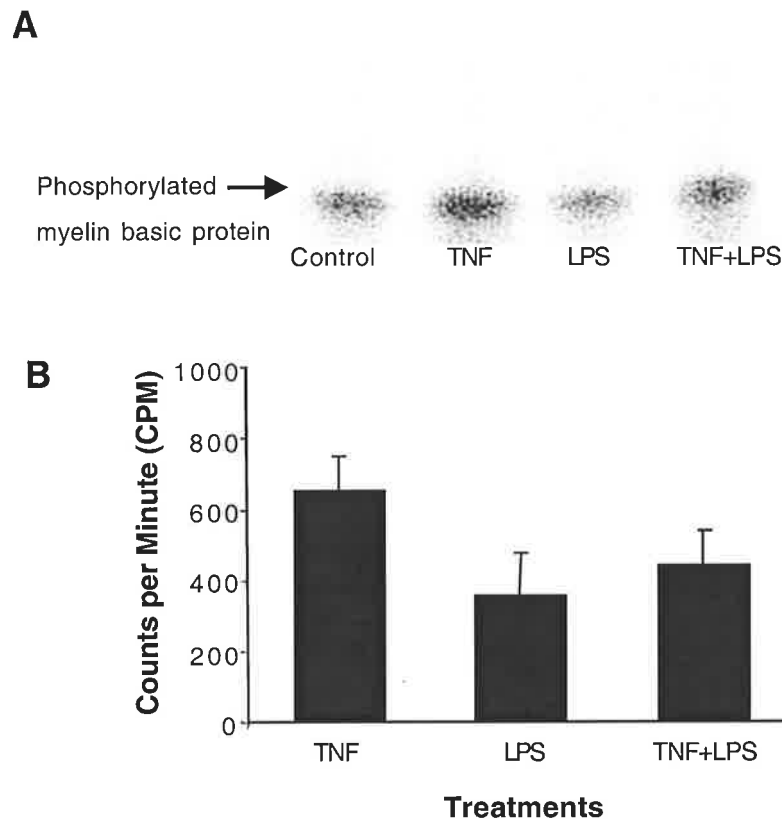
Figure 7-4:   The effects of TNF-α and LPS on the activity of ERK. HUVEC were incubated for 15min with diluent, TNF-α (25U/ml), LPS (0.1ng/ml) or TNF and LPS combined and then processed as described in material and methods. The results represent counts per minute of ^{32}P incorporated in myelin basic protein. **A.** A representative image of a gel obtained by an Instant Imager™ is shown. **B.** The data are expressed as counts per minute and represent the means ± SEM of 5 experiments, each conducted with cells from a different cord. The mean CPM of control cells (1327) was subtracted from the means of the other treatments. There were no statistically significant differences between treatments.

7.4.2   JNK

The activity of JNK was assessed by the incorporation of radioactivity into glutathione *S*-transferase –jun (1-79). The results show that both TNF-α and LPS stimulate JNK activity. While the treatment of cells with both mediators resulted in an increase in JNK activation compared to treatment with each stimulus individually, a synergistic response was not seen (Fig. 7-5).

A

Phosphorylated glutathione
S-transferase-jun {1-79} ➡

Control   TNF   LPS   TNF+LPS

B
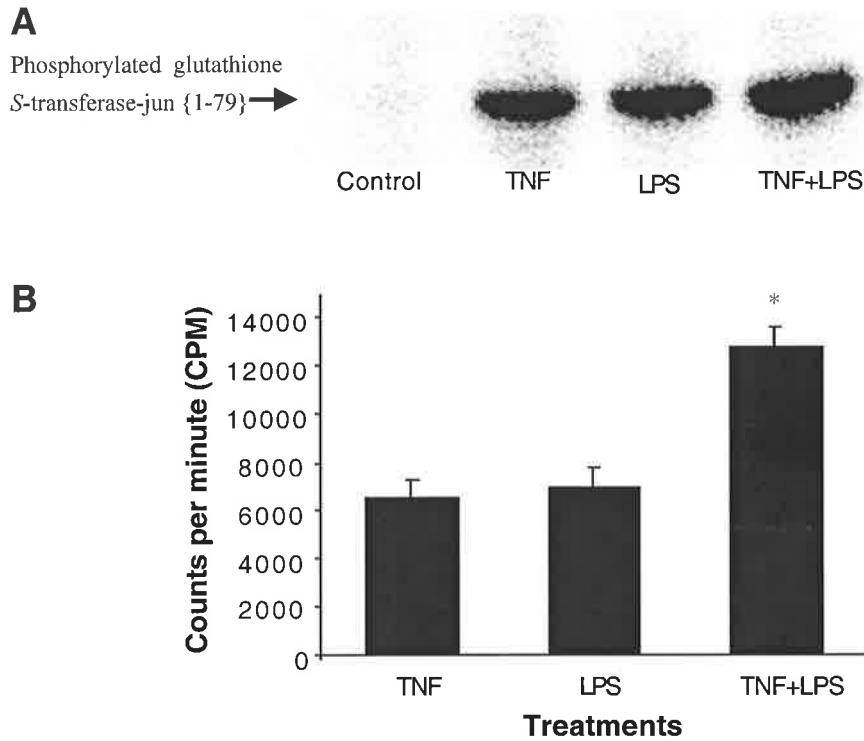
Figure 7-5:   The effects of TNF-α and LPS on the activity of JNK. HUVEC were treated as described in the legend of Fig. 7-4. **A.** A representative image of a gel obtained by Instant Imager is shown. **B.** The results are expressed as counts per minute of ^{32}P incorporated in glutathione S-transferase-jun (1-79). The data represent the means ± SEM of 5 experiments, each conducted with cells from a different cord. The baseline (520 CPM) was subtracted from the means of the other treatments. For each TNF or LPS vs TNF+LPS: $p < 0.001$. Sum of TNF and LPS vs co-stimulation (TNF+LPS): $p > 0.05$.

7.4.3   p38

The p38 activity was deterrmined by measuring the ^{32}P phosphorylation of myelin basic protein (see also 7.4.1). A representative scan is shown. (Fig. 7-6A). Co-stimulation of endothelial cells with TNF and LPS resulted in a synergistic increase in the activity of p38 (Fig. 7-6B).

A

Phosphorylated ⟶
myelin basic protein

Control   TNF   LPS   TNF+LPS

B
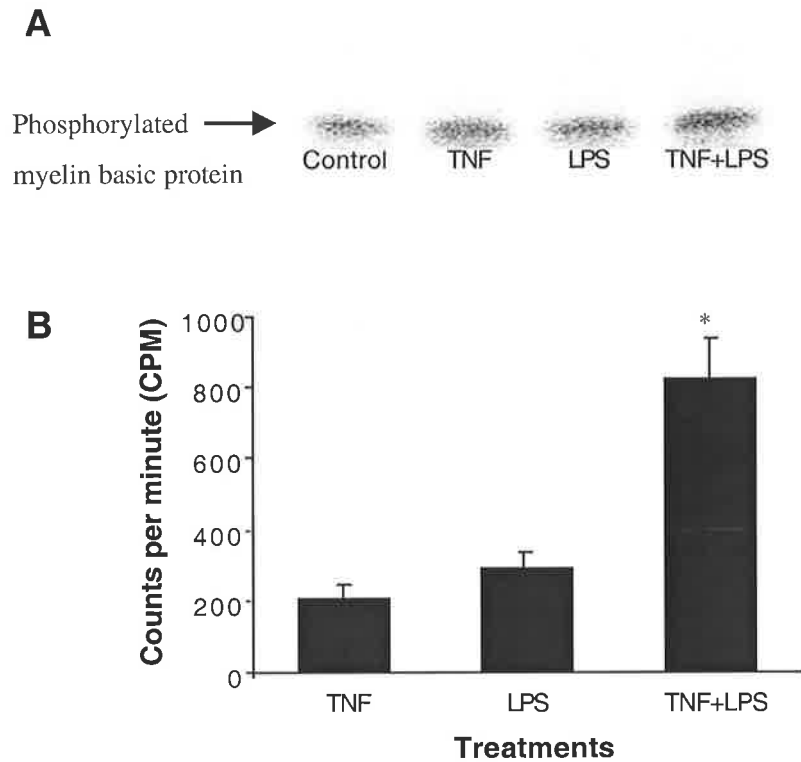
Figure 7-6:   The effects of TNF-α and LPS on the activity of p38. HUVEC were treated as described in the legend of Fig. 7-4. **A.** A representative image of a gel obtained by an Instant Imager™ is shown. **B.** The results represent counts per minute of ^{32}P incorporated in myelin basic protein. The means ± SEM of 5 experiments, each conducted with cells from a different cord, are shown. The baseline (1306 CPM) for diluent-treated cells has been subtracted from the means of the radioactivity of all treatments. TNF or LPS vs TNF+LPS: $p < 0.01$; sum of TNF and LPS vs co-stimulation (TNF+LPS): $p < 0.05$.

7.5   THE EFFECT OF CERAMIDE ON TNF-α- AND LPS-INDUCED E-SELECTIN EXPRESSION IN HUVEC

It has been suggested that ceramide mimics LPS effects. It was therefore of interest to test this hypothesis in our system. HUVEC were grown to confluence in 96-well plates (5×10^4 cells/well) and treated in quadruplicate with diluent, TNF-α (25U/ml), LPS (0.1ng/ml) or TNF+LPS, in the absence or presence of cell-permeable ceramide (C_6, 5μM). After 5.5h E-selectin expression was assessed by ELISA (as described in Methods). Ceramide alone did not stimulate E-selectin expression. Ceramide enhanced the upregulation of E-selectin caused by each of the treatments, TNF-α, LPS and TNF+LPS (Fig. 7-7). Conducting the experiments with a lower dose of ceramide (1μM) yielded similar results (data not presented). With both of these ceramide concentrations the HUVEC monolayers remained intact, whereas using a higher dose (10μM) caused disruption of the monolayer and loss of cells (data not presented).
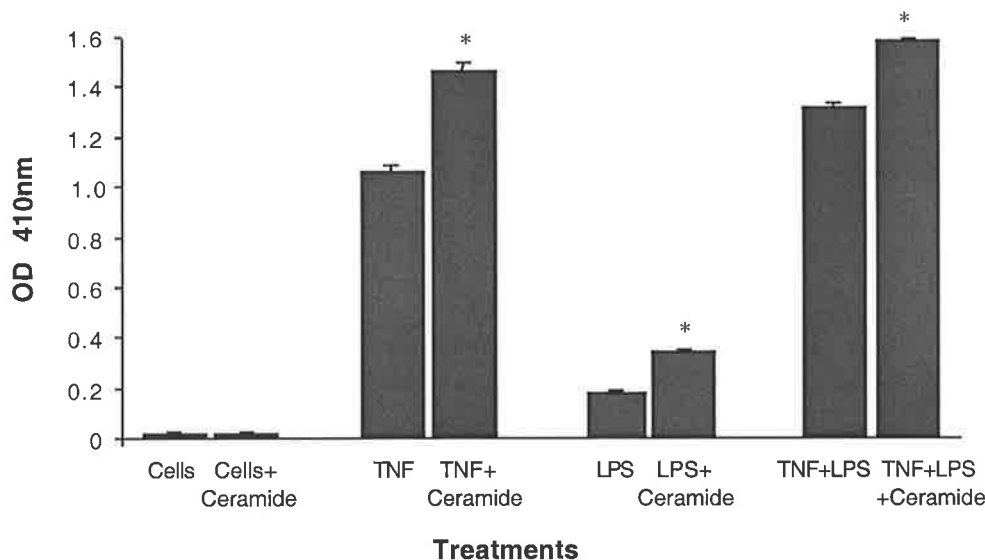
Figure 7-7: Effect of extracellular ceramide on the E-selectin expression in endothelial cells. HUVEC (5×10^4 cells/well) were treated in 1% serum with either diluent, TNF-α, LPS or TNF and LPS in the absence or presence of ceramide (C_6, 5μM) for 5.5h. There was no detectable E-selectin expression with ceramide alone. * $p < 0.001$ for TNF vs TNF+Ceramide, LPS vs LPS+Ceramide and TNF+LPS vs TNF+LPS+Ceramide.

7.6   SUMMARY AND CONCLUSIONS

TNF-α and LPS were synergistic in expression of adhesion molecule mRNA. TNF-α and LPS also caused a profound synergistic activation of the transcriptional factor NF-κB. Particularly interesting was the observation that in the presence of both TNF-α and LPS the normally transient activation of NF-κB persisted for as long as 28h.

When the activities of other relevant intracellular signalling molecules, p38, JNK and ERK, were investigated, TNF-α and LPS were synergistic in the upregulation of the activity of p38. The activity of JNK was increased in an additive manner. While both mediators individually increased the activity of ERK, which was constituively expressed in resting endothelial cells, there was no enhancement with costimulation.

While extracellular ceramide did not mimic the ability of LPS to induce upregulation of E-selectin and had no effect on adhesion molecule expression by itself, it was able to enhance the upregulation of E-selectin induced by other agonists.

These data suggest that joint activation of the signalling molecules NF-κB, p38, and JNK but not ERK are likely to be the basis for the synergism between TNF-α and LPS in the upregulation of endothelial adhesion molecule expression.

Chapter 8

**The LPS Receptor CD14 on Leukocytes,
Endothelial and Respiratory Epithelial Cells**

8.1   INTRODUCTION

The LPS homing receptor CD14 is expressed by monocytes at high levels. These cells are the source of most of the sCD14 found in human serum (Van Vorhis et al., 1983; Bazil et al., 1986). There is evidence for a high CD14-turnover rate, since treatment of unstimulated monocytes with the translation inhibitor actinomycin D leads to the complete disappearance of surface CD14 (Kruger et al., 1996). The effects of various mediators on the expression of CD14 by monocytes have been described. IL-4, IFN-γ, GM-CSF and PMA all downregulate CD14 expression over a period of 48-72h (Lauener et al., 1990; Bazil et al., 1991; Kruger et al., 1996). In contrast, LPS at concentrations of 0.1-1ng/ml upregulates monocyte expression of CD14 with a maximal effect seen at 48h. However, concentrations of LPS of 100ng/ml or higher cause CD14 to be shed (Landmann et al., 1991). Interestingly, concurrent treatment of monocytes with LPS (0.5ng/ml) prevents GM-CSF-induced down-regulation of CD14. TNF-α (20pg/ml-5ng/ml) had no influence on monocyte CD14 expression after 48h of treatment (Kruger et al., 1996).

In contrast to monocytes and macrophages, neutrophils express low levels of CD14 on their surface. Nevertheless, neutrophils respond to low doses of LPS in a CD14-dependent manner, both in vivo and in vitro (Wright et al., 1991). LPS stimulates neutrophil adhesion to protein-coated surfaces, respiratory burst and microbicidal activity (Cohn et al., 1960; Dahinden et al., 1983; Guthrie et al., 1984). The number of CD14 molecules on the neutrophil surface is estimated to be of the order of 5,000 per cell and can be up-regulated by various agonists such as G-CSF, GM-CSF, fMLP and TNF-α. The increase is complete within 20min and modest in magnitude, ranging from 1.6-fold to 2.0-fold (Wright et al., 1991). However, this important observation has not previously been correlated to the LPS-induced neutrophil functions.

While endothelial cells are sensitive to low concentrations of LPS (Chapter 5), no evidence has been presented to date to suggest that CD14 is found on these cells and it has been generally accepted that endothelial cells do not express CD14 (Beekhuizen et al., 1991). In the absence of mCD14 the soluble form of the antigen is thought to facilitate LPS-induced cell activation (Frey et al., 1992). Surprisingly, anti-CD14 antibodies block endothelial cell activation by LPS in the absence of serum (Von Asmuth et al., 1993). These observations taken together are inconsistent with the concept that endothelial cells do not express CD14.

There is no consensus view whether respiratory epithelial cells express CD14. While most regard these cells as CD14 negative (Cox et al., 1992; Keicho et al., 1997; Striz et al., 1998) there have been reports describing the expression of CD14 in murine lung epithelial cells (Fearns et al., 1995 and 1997). CD14 was also detected in bovine tracheal epithelial cells (Diamond et al., 1996). In addition, the expression of CD14 in epithelial cells of organs other than the lung has been described. These include those of the murine colon (Meijssen et al., 1998), human colon (Martin-Villa et al., 1997), murine kidney and murine liver (Fearns et al., 1995). Interestingly, airway epithelial cells are generally regarded as either unresponsive or hyporesponsive to LPS. Human bronchial epithelial cells (HBEC), although staining negative for CD14, bound FITC-conjugated LPS in a dose dependent manner.

Surprisingly anti-CD14 antibody did not inhibit the binding of f-LPS, nor did sCD14 enhance it (Striz et al., 1998). LPS was found to bind to rat alveolar type II cells, but the influence of anti-CD14 on this binding was not examined (Aracil et al., 1985).

8.2   RESULTS

8.2.1   Effects of TNF-α on monocyte CD14 expression

Since the results presented in Chapter 3 showed an enhancement of LPS-induced cytokine production of monocytes by TNF-α, we examined whether or not this enhancement was associated with a TNF-α-induced up-regulation of monocyte CD14 in this time frame. Peripheral blood monocytes were re-suspended in RPMI 1640 supplemented with 5% autologous heat inactivated donor serum. The cells (1×10^6/assay) were treated with TNF-α (100U/ml) for 0, 5, 15, 30, 60 and 120min and then processed for indirect immuno-fluorescence and flow cytometry analysis as described in section 2.2.13. The results showed that there was no change in monocyte CD14 expression by treatment with TNF-α compared to cells treated with media only (Fig. 8-1).
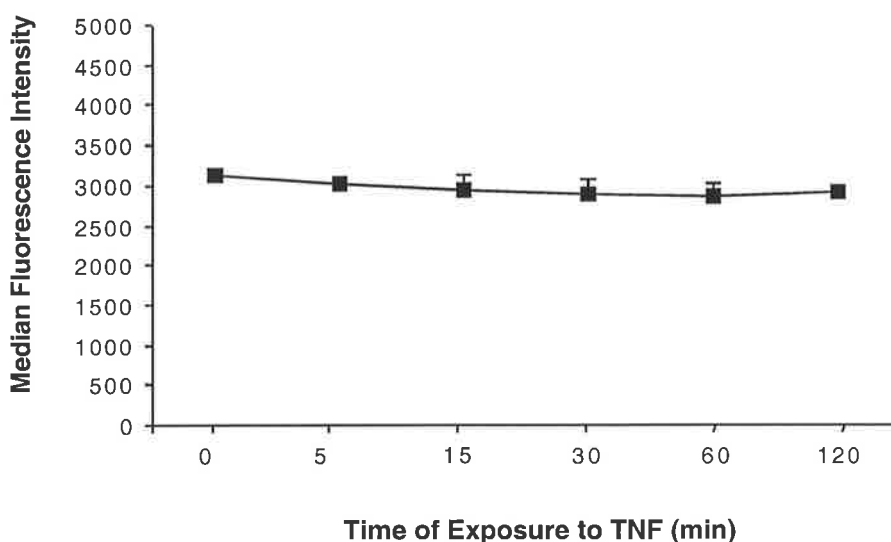
Figure 8-1:   Effect of TNF-α treatment on the CD14 expression of monocytes. Monocytes were purified from peripheral blood as described in Methods. The cells (1×10^6/assay) were re-suspended in RPMI 1640/5% FCS and treated with TNF-α (100U/ml) for the times indicated and then the expression of CD14 determined by indirect flow cytometric analysis. The data represent the means ± SEM of duplicates derived from experiments with cells from 3 different donors. The mean value for the samples stained with isotype-control antibody (18.9) has been subtracted from all treatments. There was no statistically significant difference between the data points.

Repeating the experiments using lower or higher concentrations of TNF-α (10U/ml, 1000U/ml) produced similar results (data not presented). Similar experiments in which monocytes were stimulated with LPS (0.1, 1, 10, and 100ng/ml) instead of TNF-α also did not change monocyte CD14 expression (data not presented).

8.2.2   Effects of TNF-α on the surface expression of CD14 by human neutrophils

The results presented in Chapter 4 showed that pretreatment of neutrophils with TNF-α led to synergistic enhancement of two LPS-induced effects, the neutrophil respiratory burst and the adhesion of neutrophils to serum-coated plastic. The optimal pretreatment time was found to be 15-20min, consistent with translocation of preformed molecules to the cell surface. Thus, up-regulation of surface CD14 may be one of the mechanisms by which TNF-α alters the response to LPS. To investigate this the expression of CD14 on neutrophils and the surface binding of LPS to these cells were studied using the same experimental parameters as in the experiments described in Chapter 4.

Neutrophils (1×10^6/assay) were treated with either TNF-α (20U/ml) or diluent at 37^0C for 20min, and then the expression of CD14 was quantified by flow cytometry. TNF-α pretreatment led to a 2.1-fold increase in CD14 surface expression on neutrophils (Fig. 8-2).
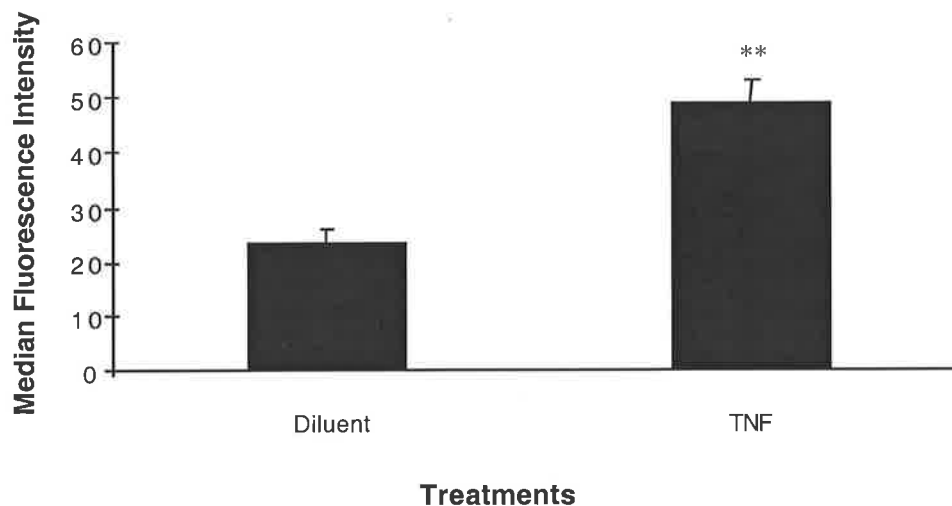
Figure 8-2:   The effect of TNF-α pre-treatment on surface CD14 on neutrophils. Neutrophils (1×10^6) were incubated with either buffer or TNF-α (20U/ml) for 20min. The cells were first incubated with anti-CD14, then washed and incubated with goat anti-mouse PE (all at 4°C). The intensity of fluorescence was determined as described in section 2.2.8. The data shown represent the means ± SEM of 5 experiments each conducted with cells from a different donor. (** = p < 0.001, student t-test).

To assess LPS binding neutrophils (1×10^6/assay) were pre-treated with either TNF-α (20U/ml) or diluent for 20min at 37^0C. Then autologous donor serum (5%, heat-inactivated) and FITC-labelled LPS (16µg/ml) were added. Control cells were exposed to FITC only, while neutrophils exposed to unlabelled LPS served as a background control (autofluorescence). The amount of LPS bound was measured by flow cytometry analysis. The results showed a 2.2 fold increase in LPS binding by neutrophils pretreated with TNF-α compared to the control. The increase in fluorescence was abrogated by incubating TNF-α-treated neutrophils with an anti-CD14 monoclonal antibody (MY4, dilution 1:10, 30min, 4^0C) prior to adding FITC-LPS (Fig. 8-3).
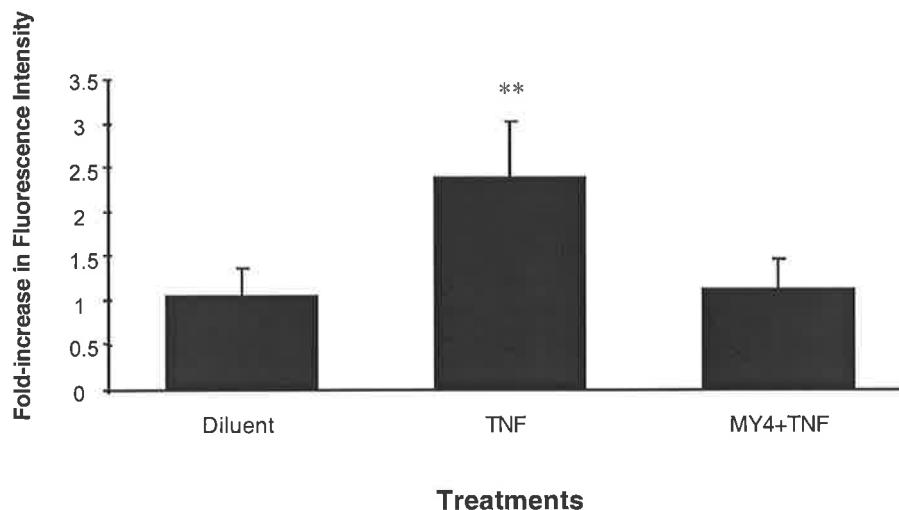
Figure 8-3:   The effect of pre-treatment with TNF-α on LPS binding to CD14. Neutrophils (1x10^6) were stimulated as described in the legend of Fig. 8-2. The cells were re-suspended in serum (5%, autologous, heat-inactivated) and incubated with FITC or FITC-labelled LPS (*E. coli* 0127:B8) at a final concentration of 16μg/ml for 30min on ice. Some cells were treated with MY4 (1:10, 30min) prior to the addition of f-LPS. Flow cytometry was performed as described in section 2.2.8. The data shown are the means ± SEM of 5 experiments each conducted with cells from a different donor. The values of cells treated with FITC only have been subtracted from the treatments. (TNF vs Diluent or MY4+TNF, ** = p < 0.01, ANOVA).

8.2.3   Human Umbilical Vein Endothelial Cells

8.2.3.1   Expression of CD14 on HUVEC

Based on inconsistent published data (8.1) we predicted that contrary to current belief endothelial cell express CD14, perhaps at low levels, and that this expression had been overlooked in the past. Assay conditions for detecting CD14 expression were examined using monocytes since high expression of CD14 on these cells has been well established. To standardize the assay, five different anti-CD14 monoclonal antibodies were tested for their degree of staining of monocytes using PE-conjugated secondary antibody (Fig. 8-6A). With optimal concentrations of these antibodies it was evident that MY4 was the most effective monoclonal antibody for detection of CD14 expression. MY4 was therefore used in all subsequent experiments unless stated otherwise. Endothelial cells stained with MY4 displayed a median fluorescence intensity (MFI) distinct from that of cells treated with the isotype-matched control antibody (Fig. 8-4B and Fig. 8-5).
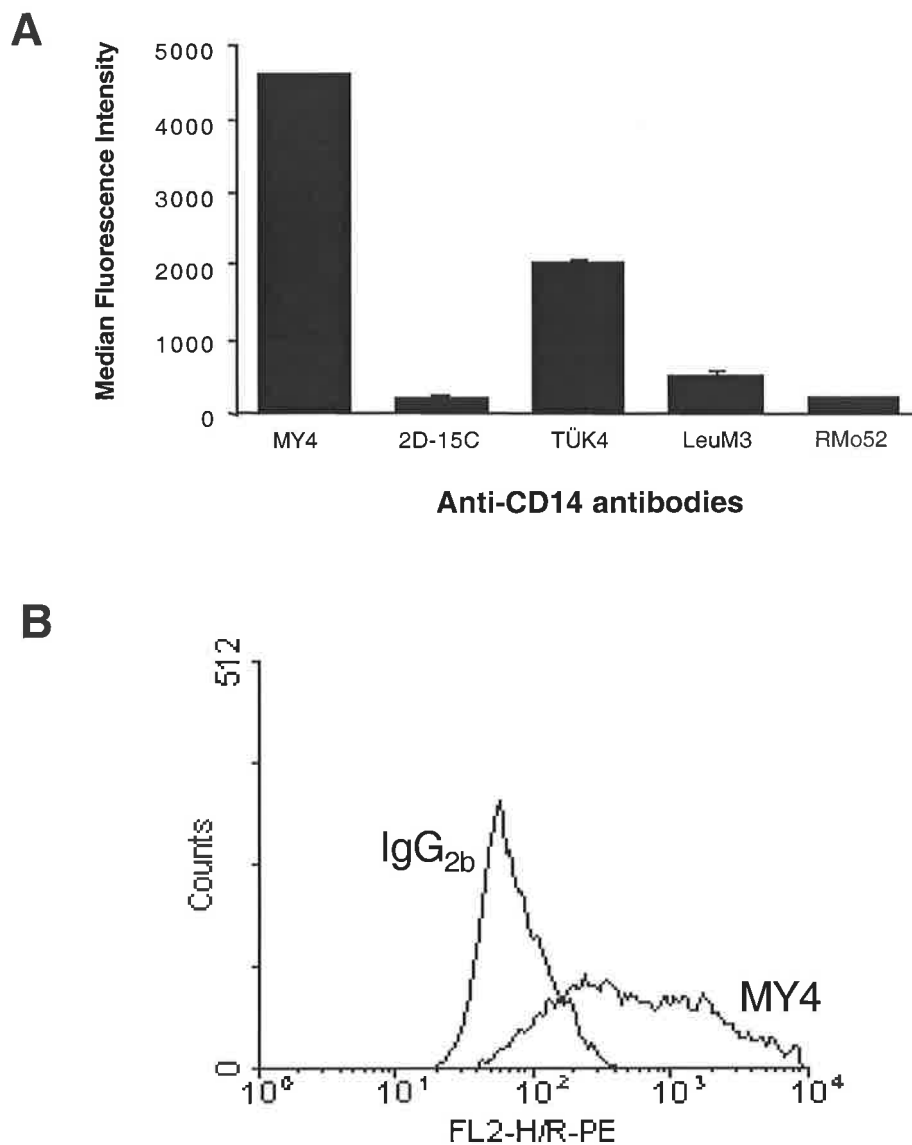
Figure 8-4:   Expression of CD14 on endothelial cells. **A.** Monocytes were stained for indirect immuno-fluorescence using different primary monoclonal antibodies against CD14 (1:50) and a rabbit anti-mouse R-PE-conjugated polyclonal antibody (1:100) as the secondary antibody. **B.** A typical histogramme of HUVEC stained by the indirect fluorescence method illustrates the shift in FL-2 fluorescence of cell populations incubated with anti-CD14 antibody (MY4) when compared with isotype control antibody (IgG$_{2b}$).
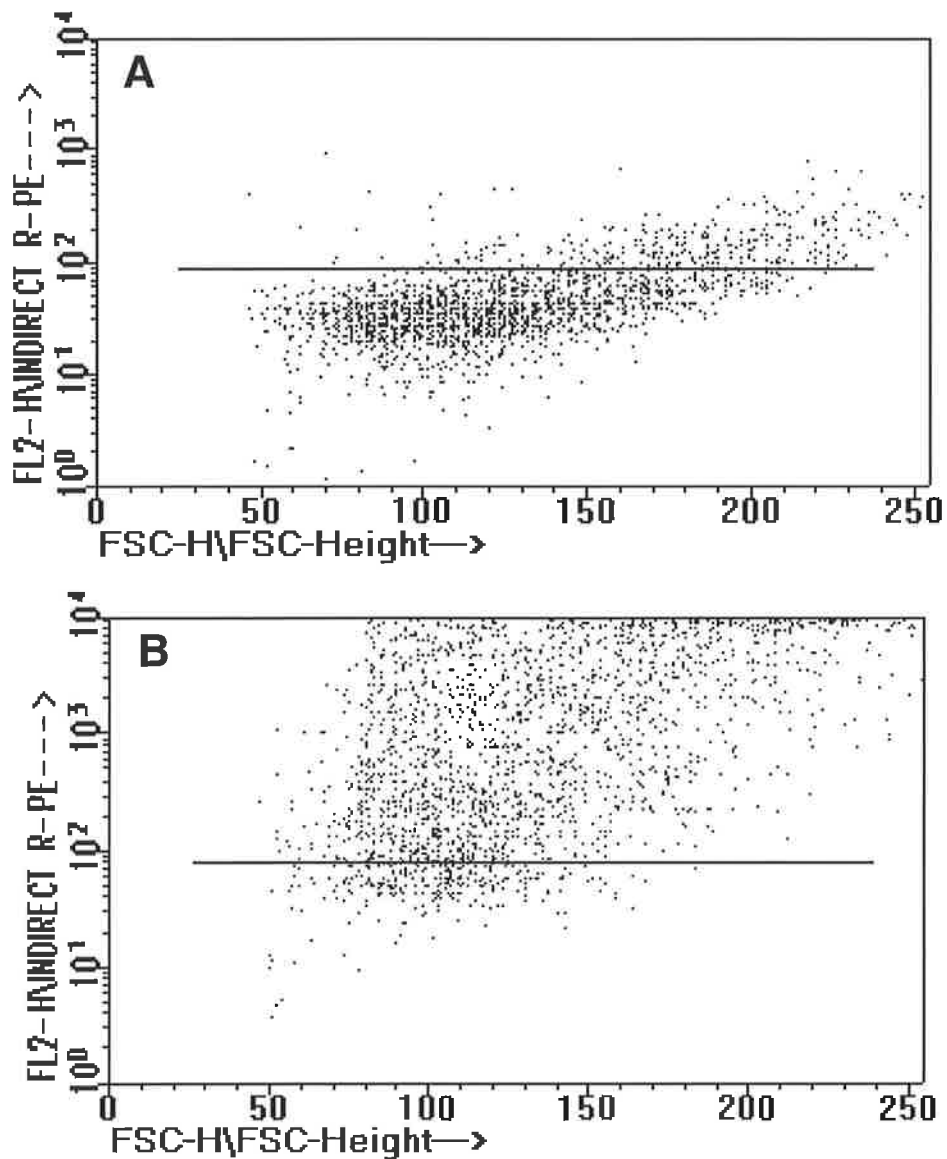
Figure 8-5: Expression of CD14 on endothelial cells. Representative dot-plot diagrams of HUVEC $(1 \times 10^5/\text{assay})$ stained with **A.** IgG_{2b} isotype control antibody or **B.** anti-CD14 antibody MY4 (both 1:50) are shown. The secondary antibody was a rabbit anti-mouse R-PE-conjugated polyclonal antibody (1:100). The events (10,000 each) are plotted flow channel two (FL2, R-PE) against forward scatter (FSC).

HUVEC, grown to confluence on slides, stained positive by indirect immunofluorescence microscopy using MY4 (Fig. 8-6A), as did the endothelial layer of umbilical veins *in situ* (Fig. 8-6B). There was no staining of cell outlines or of the endothelial layers of the cord vessels when IgG_{2b} isotype-matched control antibody was used as the primary antibody (not shown).
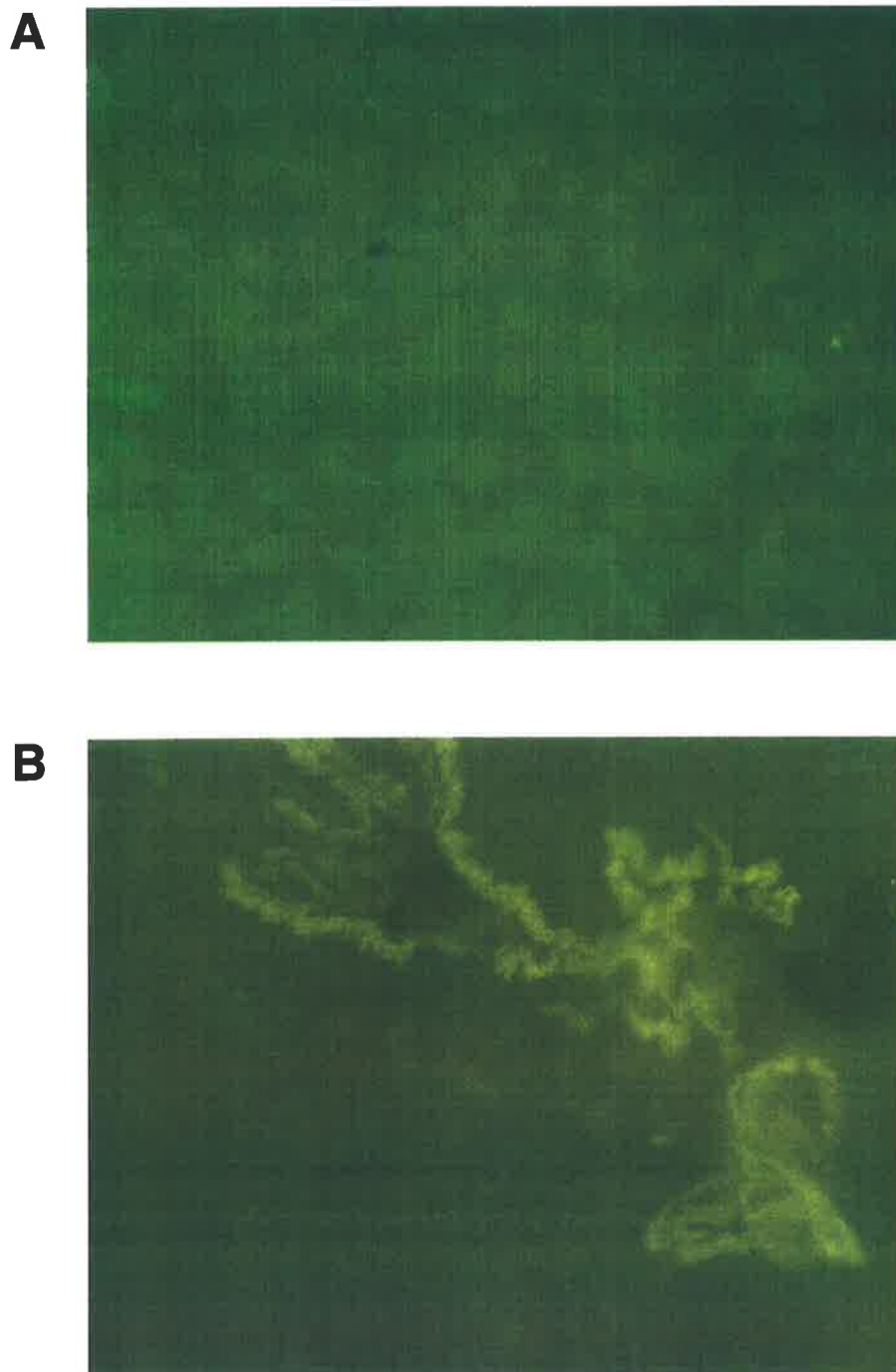
Figure 8-6:   MY4 stains HUVEC in culture and *in situ*. **A.** HUVEC were grown on slides and then fixed. Staining was performed with MY4 primary antibody and FITC-conjugated rabbit-anti mouse polyclonal secondary antibody (magnification: 100x). **B.** Cords were snap-frozen, cut, transferred onto slides and stained as described above. The endothelial layer of the partially collapsed umbilical vein displayed bright fluorescence (magnification: 10x).

8.2.3.2   Specificity of MY4

MY4 displayed much better staining ability compared to other anti-CD14 antibodies. To show MY4 was specific for CD14 and to rule out that it may bind to another surface antigen different from CD14 the following experiments were carried out:

A. Untreated HUVEC were enzymatically removed from the culture dish. The cells were incubated with diluent, IgG_{2b} isotype-matched control antibody, or MY4 (1:25), and then washed, incubated with biotinylated IgG_{2b} or MY4 (both 1:100), washed again and finally incubated with streptavidin-PE (SA-PE, 1:100). Unconjugated MY4 was able to prevent its biotinylated form from binding. Non-specific IgG_{2b} had no effect (Fig. 8-7A).

B. HUVEC were pre-incubated with diluent, IgG_{2b}, or TÜK4 (both 1:10) and after washing treated with either IgG_{2b}-PE or TÜK-PE (both 1:50). The results achieved with MY4 could thus be repeated with an antibody raised against a different epitope of CD14 (Fig. 8-7B).

C. HUVEC were prepared for flow cytometry and resuspended in RPMI 1640 containing increasing amounts of heat-inactivated, pooled human group AB serum and then stained with MY4 (1:1500). Serum was able to significantly reduce staining of endothelial cells by MY4 in a concentration-dependent manner (Fig. 8-7C), suggesting that a serum factor was neutralizing the anti-CD14 monoclonal antibody. This factor is likely to be sCD14.
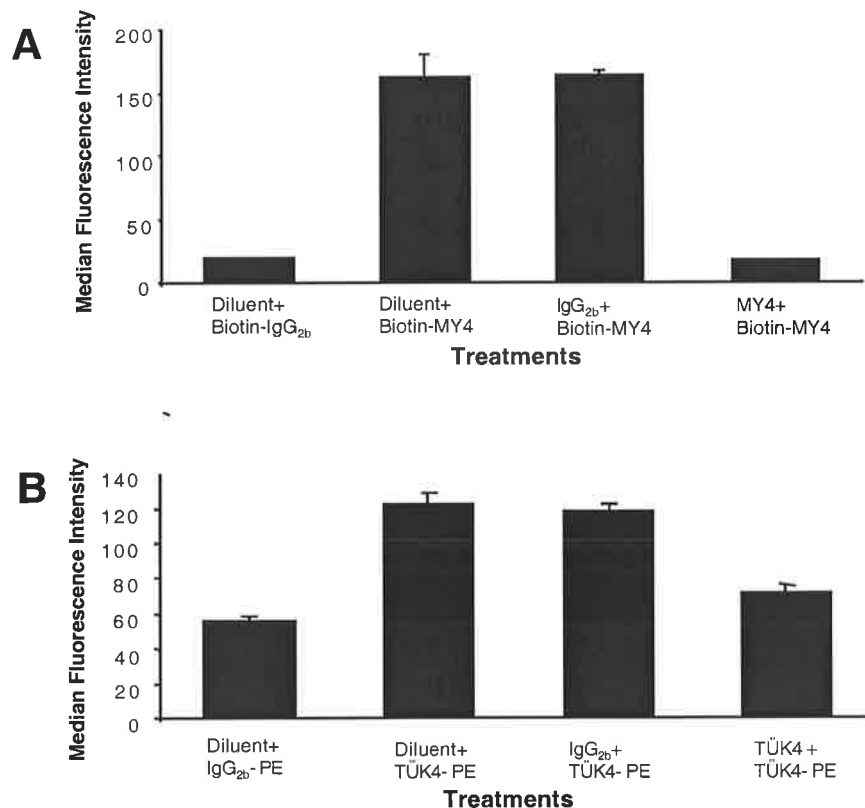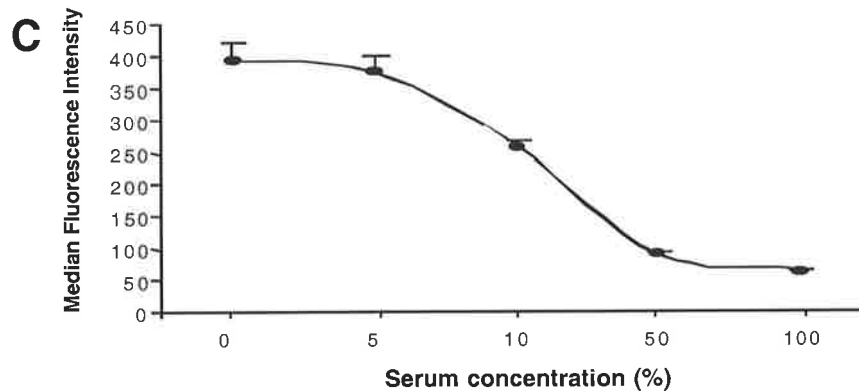
Figure 8-7: MY4 is specific for CD14. **A.** Unconjugated MY4 prevents biotinylated MY4 from staining HUVEC, IgG$_{2b}$ does not (visualized with streptavidin-PE). **B.** TÜK4 prevents TÜK4-PE from staining HUVEC. C. Increasing amounts of AB serum significantly reduce binding of MY4 (1:1500) to HUVEC. All data represent the means ± SEM of duplicates from experiments with 2 different cords.

8.2.3.3 Assessment of HUVEC cultures for monocyte contamination

HUVEC prepared from fresh umbilical cords may be contaminated with monocytes. The degree of such contamination was investigated by co-staining endothelial cells prepared for flow cytometry with the white cell marker CD45, which is not expressed by endothelial cells. Consistently less than 0.01% of the viable cell population stained positive (Fig. 8-8).
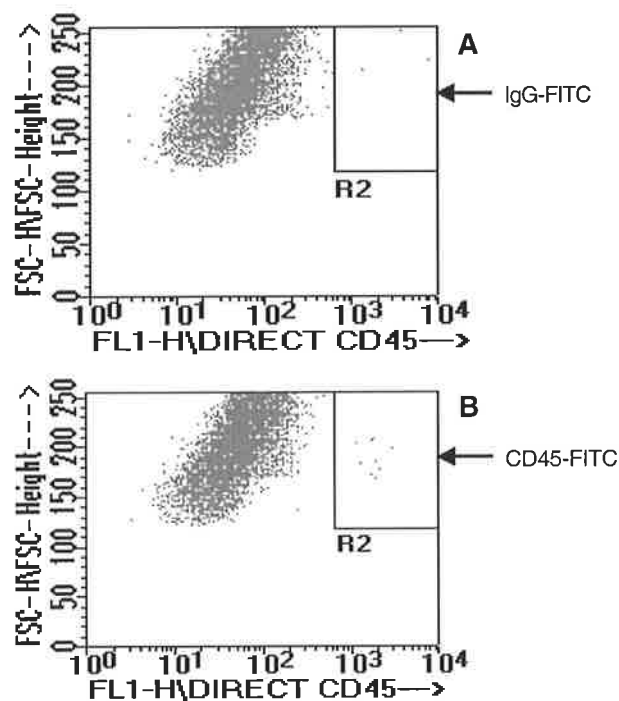
Figure 8-8: Assessment of monocyte contamination of HUVEC cultures. Representative dot-plots of HUVEC stained with either isotype control-matched FITC-conjugated antibody (**A**) or anti-CD45-FITC (**B**) are shown. The FITC-positive cells have been gated (R2). The events in gate R2 for the isotype control antibody were subtracted from the events for the CD45 antibody. Less than 0.01% of the cell population of 10,000 were CD45 positive. These cells are most likely to be monocytes, since they also stained very brightly for CD14 R-PE (not shown).

Similar results were achieved by using antibodies against other white cell antigens, CD11b and CD33 (results not shown). Furthermore the patterns of the histograms and dot plots obtained when staining HUVEC with MY4 are not consistent with the CD14-positivity of these cells being merely an artifact due to monocyte contamination (Fig. 8-4B and Fig. 8-5).

8.2.3.4 Loss of cell surface expression of CD14 during subculturing

Routine passaging of primary cultures of HUVEC or purchasing HUVEC from tissue culture laboratories at passages 3 to 5 is widely practiced. It was therefore of interest to investigate whether the ability of HUVEC to express CD14 on the surface was dependent on the number of passages the endothelial cells had been subjected to. When HUVEC were grown in culture medium containing FCS (10%), heparin (2.5%) and ECGS (150μg/ml) and passaged every 3 days, the cells rapidly lost CD14 with subculturing. By passage 3, CD14 expression was less than 14% of the expression of passage 1 cells and less than 7% by passage 7 (Fig. 8-9). Thus, in subsequent experiments passage 1 cells were used unless otherwise stated.
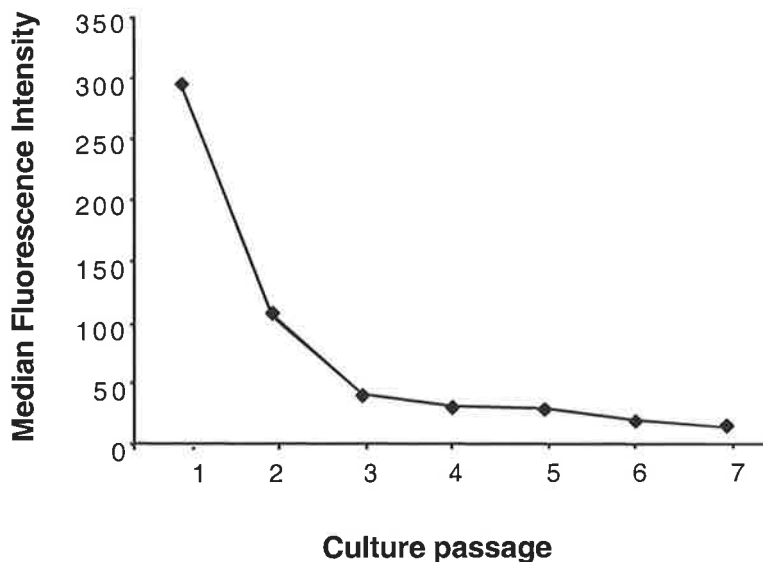
Figure 8-9:   Effect of passaging HUVEC on CD14 expression. HUVEC were grown in RPMI 1640 medium supplemented with FCS, heparin and ECGS. Every 3 days the cells were passaged, at which point one third of the cells were prepared for flow cytometric analysis. The data represent the means of 3 experiments conducted with cells from different umbilical cords.

In the light of these findings, it was of interest to examine a frequently used human endothelial cell line (HUV-EC-C) which is repeatedly subcultured (lifetime ~50-60 passages) and also propagated in FCS/ECGS/heparin-supplemented medium, as to whether these cells expressed CD14. The level of CD14 expression by HUV-EC-C was very low, resembling the expression displayed by primary cultures of HUVEC at passage 6 (Fig. 8-10).
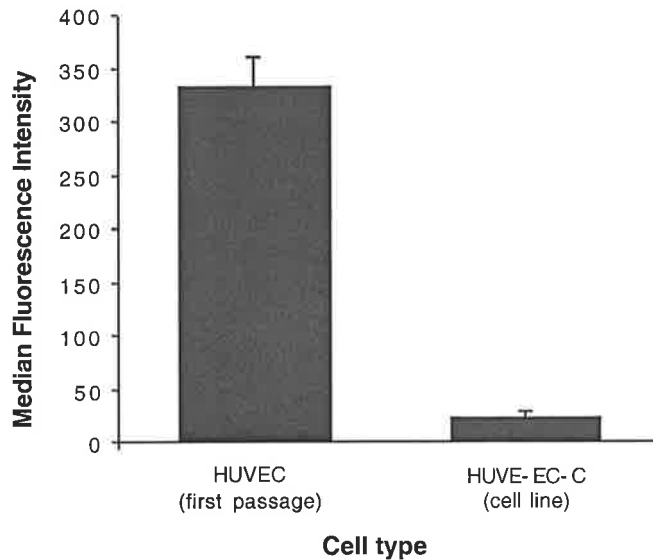
Figure 8-10:   Level of expression of CD14 by HUVE-EC-C. This human endothelial cell line was examined at passage 15 (normal life expectancy 50-60 passages). The expression of CD14 was very low, similar to primary HUVEC at passage 6. The data are the means ± SEM of duplicates of 2 independent experiments.

8.2.3.5 LPS-binding to endothelial cells

It has been described that LPS does not bind to endothelial cells. Encouraged by the demonstration of CD14 on the cell surface of passage 1 cells and the hypothesis that the loss of CD14 expression during passaging might have accounted for that result, an attempt was made to stain first-passage HUVEC with FITC-conjugated LPS (f-LPS). There was no statistically significant difference in MFI between HUVEC incubated with f-LPS or unlabelled LPS (data not shown).

8.2.3.6 Estimation of the number of CD14 expressed on HUVEC

In order to estimate the number of CD14 molecules expressed per endothelial cell two approaches were taken. Firstly, peripheral blood monocytes from 12 healthy donors were examined under the same conditions as endothelial cells obtained from 12 different umbilical cords. These cells were then analyzed using identical flow cytometry instrument settings. The mean difference in MFI between the cells treated with anti-CD14 monoclonal antibody and those treated with isotype-matched control antibody was 333±28 for endothelial cells and 4,568±121 for monocytes (Fig. 8-11A).

Assuming each monocyte expresses between 29,000 and 42,000 CD14 molecules (Vasselon et al., 1997; Van Voorhis et al., 1983), then the calculated number of CD14 per HUVEC ranged from 2114 ± 400 to 3061 ± 400. Secondly, duplicates of the monocytes and HUVEC described above were stained with FITC-conjugated secondary antibody to enable their MFI to be compared to a normogram generated by analyzing repeated samples of FITC Quantum™ 26 beads. This is a middle level kit

consisting of five populations of beads of identical size, each of which coated with different, defined numbers of fluorochromes (Figure 8-11B,C). The estimates for the number of fluorochromes per cell for endothelial cells ranged from 2,000-3,000.
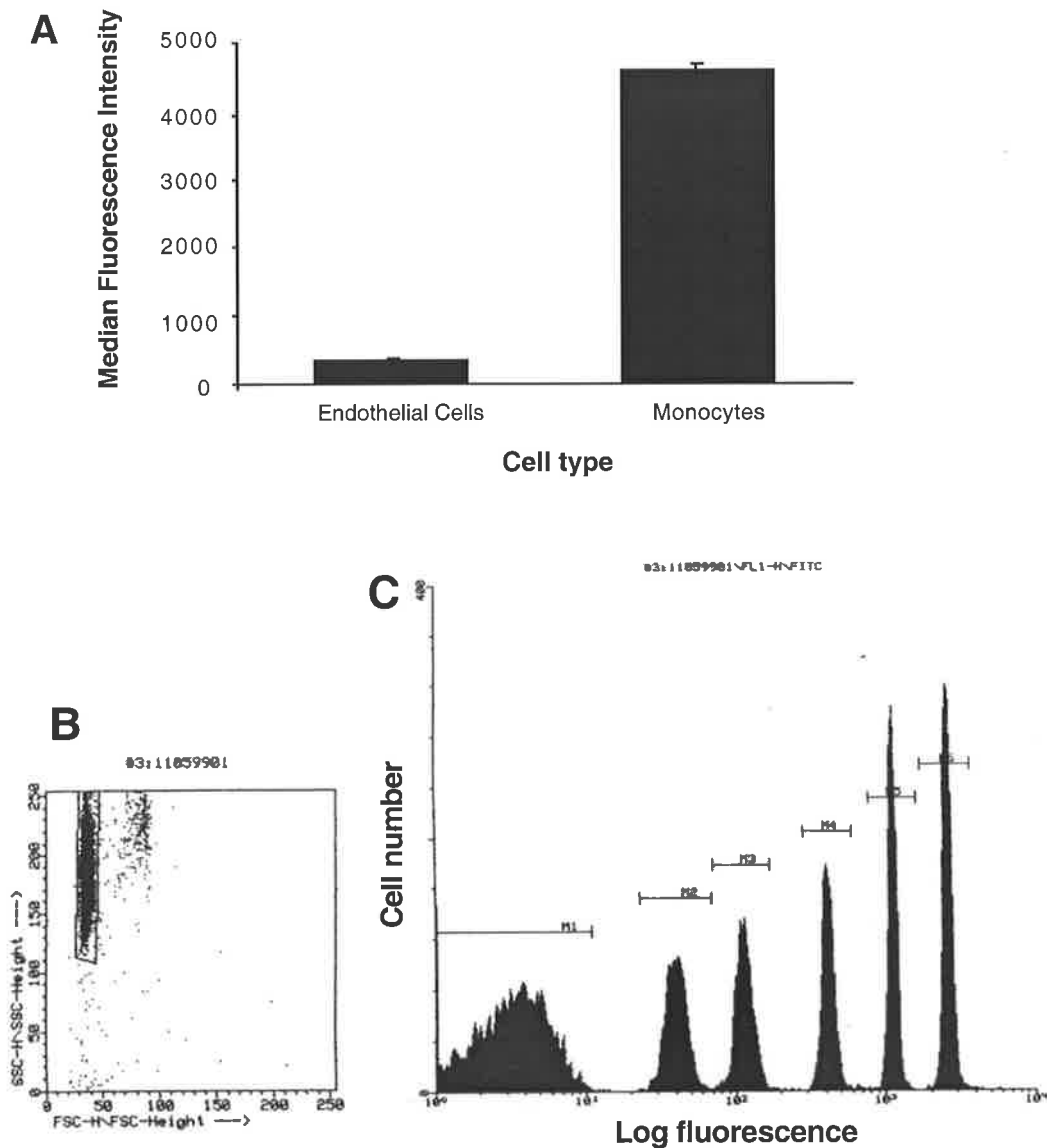
Figure 8-11:   Estimation of the number of CD14 on HUVEC. **A.** Direct comparison of CD14 brightness of HUVEC to monocytes with identical flow cytometer settings. The MFIs of passage 1 endothelial cells from 12 different cords was compared to the MFIs of monocytes (12 preparations, each from a different healthy donor). **B.** Dot-plot of FITC Quantum™ 26 beads. The gate is drawn around the singlet, non-aggregated bead population. **C.** Histogramme derived from that gate, depicting the 6 different bead populations, 1 without fluorescence (left) and 5 with given increasing amounts of fluorochrome.

8.2.3.7 Modulation of CD14 expression on HUVEC and dependence on protein synthesis

Alterations in CD14 expression as a result of the action of various mediators would not only serve as indirect evidence for the synthesis of CD14 by these cells, but may also be of significance in the sense that it may lead to changes in the responsiveness of endothelial cells to LPS. We therefore investigated whether CD14 expression on endothelial cells was amenable to alteration by cell agonists and tissue culture media supplements. The surface expression of CD14 was increased by treatment with LPS (1ng/ml), LPA (2.5µM), ECGS (150µg/ml) and FCS (40%), whereas PMA (10nM) reduced its expression (Fig. 8-12A).
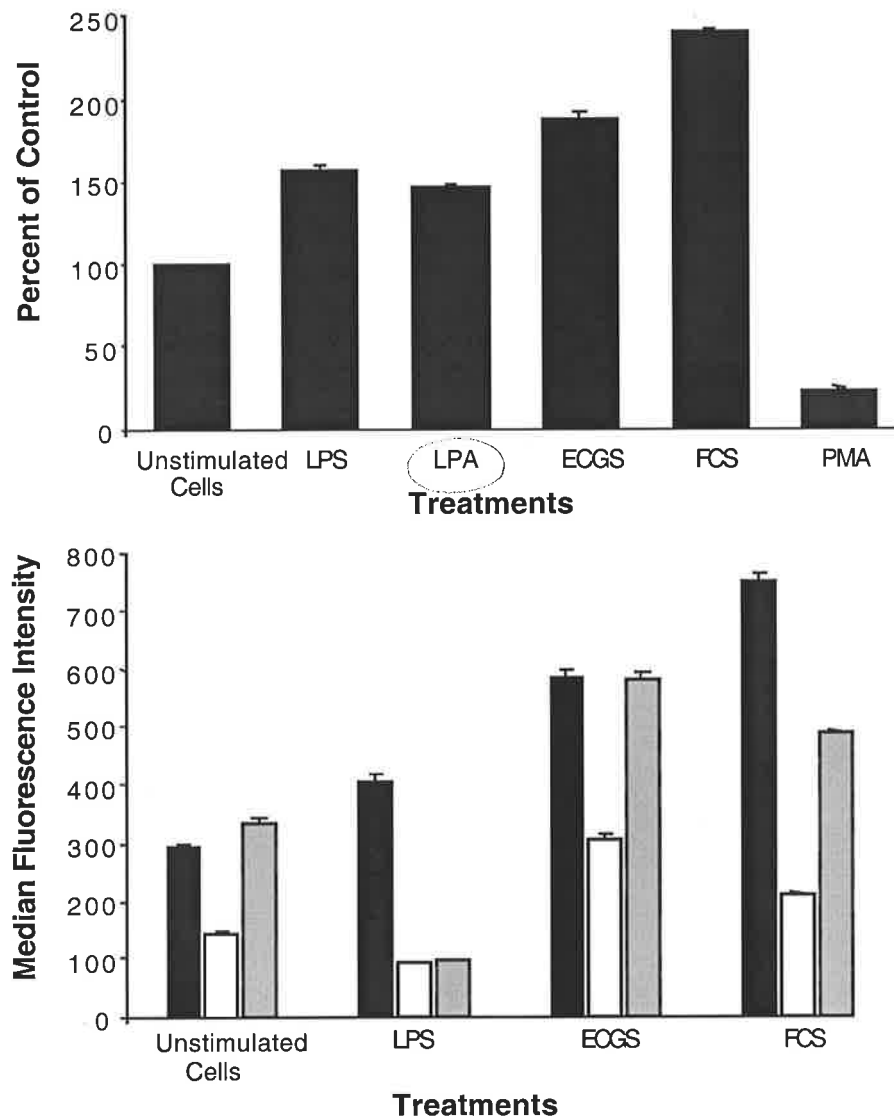
Figure 8-12:   Modulation of surface expression and evidence for synthesis of CD14 by endothelial cells. **A.** Endothelial cells were treated for 24h with LPS (1ng/ml), LPA (2.5µM), ECGS (150µg/ml), FCS (40%) and PMA (10nM). The results are expressed as percent of control; unstimulated cells with a MFI of 333 were defined as 100%. **B.** Cells were treated either with medium only (black bars) or medium with CHX (white bars), and then tested for expression of CD14 following stimulation with LPS, ECGS or FCS (same concentrations as above). After removing CHX the cells were replenished with normal culture medium and grown for 72h (grey bars). The results represent the means ± SEM of duplicates of 3 experiments with cells from 3 different cords.

LPA = Lisophosphatidic acid

When human AB-group serum was used instead of FCS at concentrations from 10%-60% there was no change in CD14 expression (data not presented). TNF-α and IFN-γ, which increase CD14 expression in neutrophils (Van Voorhis et al., 1983; Takeshita et al., 1998), had no effect on the expression of this molecule in endothelial cells (data not presented).

To exclude the possibility that CD14 expressed on endothelial cells originated from an exogenous source, HUVEC were incubated with various mediators in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX). When cells were analyzed after 24h, CHX (5μg/ml) had caused a reduction in the expression of CD14 in unstimulated cells and in LPS-, ECGS- and FCS-treated HUVEC. After the cells were washed and maintained for a further 72h in CHX-free culture medium, these effects were reversed with the exception of cells treated with LPS and CHX (Fig. 8-12B). CHX-exposed control-, LPS- or FCS-treated cells were >85% viable at the time of FACS analysis, ECGS-treated cells were >95% viable as assessed by 7-AAD staining.

8.2.3.8   CD14 is synthesized by endothelial cells

Evidence that HUVEC synthesize CD14 was derived from studies with incorporation of radio-label. Human CD14 consists of 356 amino acids, 6 of which are methionine (Ferrero et al., 1990). After incubation with ^{35}S L-methionine for 24h there was incorporation of label into CD14 in unstimulated HUVEC. Cells grown in FCS incorporated 2.3 -fold more label (Fig. 8-13A), which corresponded to the 2.3-fold increase seen in upregulation of CD14 surface expression by FCS (Figs. 8-12A,B). To confirm the proteins molecular weight immune precipitates were also subjected to SDS-PAGE. Radioactive bands of a molecular weight of 55kD were observed, consistent with the protein being CD14. The density of the bands was increased in cells stimulated with 40% FCS as opposed to unstimulated cells (Fig. 8-13B).
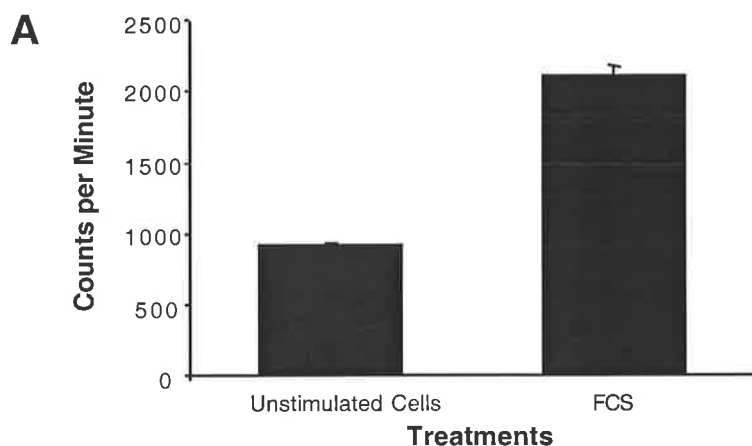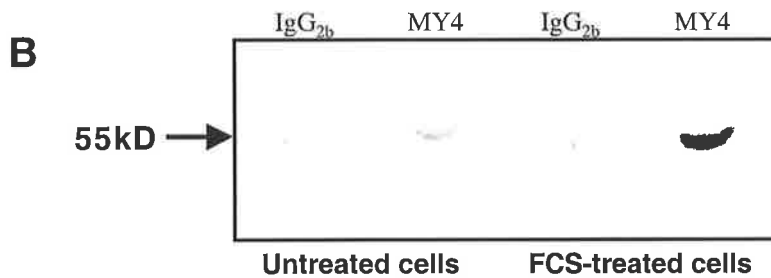
Figure 8-13:   Incorporation of radio-labeled amino acid into CD14. **A.** HUVEC were incubated for 24h with ^{35}S L-methionine and the extent of incorporation into CD14 was determined by immuno-precipitation. The results are expressed as CPM (CPM values of isotype-matched controls have been subtracted) and represent the means ± SEM of 2 independent experiments with cells from 2 different cords. **B.** The immuno-precipitates of untreated cells and cells incubated with FCS were subjected to gel electrophoresis. The bands have a MW of ~ 55 kD as determined by the migration of unlabelled markers with known MW.

8.2.3.9 Role of serum in the activation of endothelial cells by LPS

Although responsiveness of endothelial cells to LPS under serum-free conditions has been previously described (Von Asmuth et al., 1993), it is a generally held view that these cells require serum factors to respond to LPS. It was therefore important to test the response of endothelial cells in the absence of serum in our experimental settings. HUVEC were grown to confluence in 96-well plates and E-selectin expression measured by ELISA was chosen as an indicator of cell activation. The cells were treated for 6h with increasing concentrations of LPS in the presence and absence of serum. The data showed that HUVEC were activated by LPS under serum-free conditions, albeit in the absence of serum 100-fold higher concentrations of LPS were needed to achieve similar levels of E-selectin expression (Fig. 8-14).
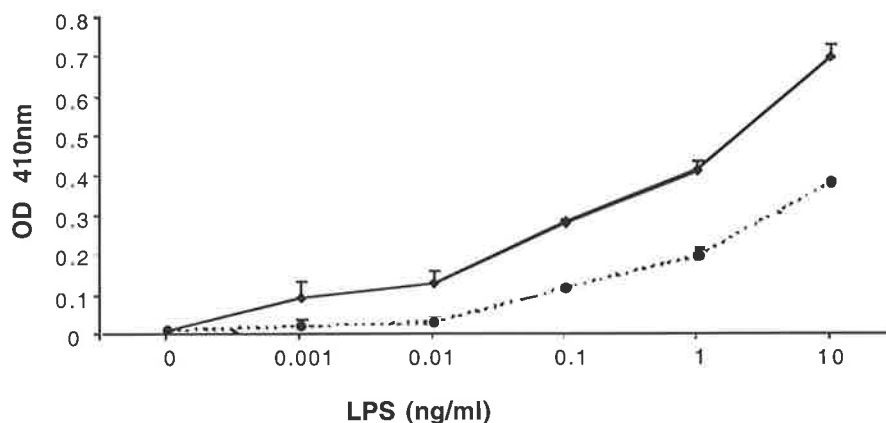
Figure 8-14:   LPS activation of endothelial cells in the presence and absence of serum. HUVEC (5×10^4/well) were grown to confluence in 96-well-plates and prior to treatment the cells were washed 4 times with RPMI 1640. The cells were then treated with varying concentrations of LPS, either in the presence of 10% human AB serum (————) or RPMI 1640 only (----------). After 6h of incubation E-selectin expression was measured by 2-step ELISA. The data are presented as the means ± SEM of quadruplicates of 3 experiments, each conducted with cells from a different cord.

8.2.3.10   Anti-CD14 antibody blocks the LPS-induced endothelial cell activation in the absence of serum

After demonstrating that HUVEC responded to LPS in the absence of serum it was important to ascertain whether this response was dependent on CD14. HUVEC were treated for 6h with diluent or LPS (1ng/ml) in the presence and absence of serum (10% AB), and in the presence or absence of MY4 (dilution 1:25). Upregulation of E-selectin by LPS was abrogated by pretreatment of HUVEC with MY4 (15min), whether serum was present or not (Fig. 8-15).
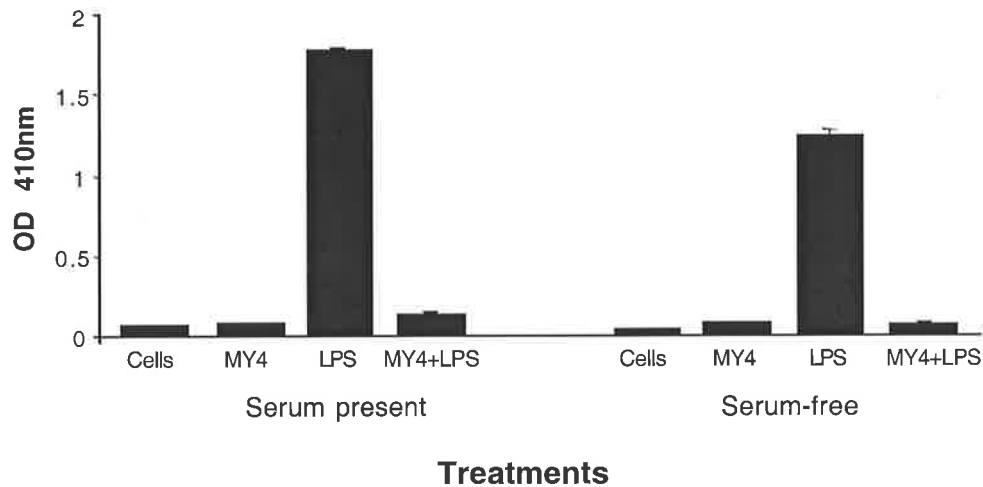
Figure 8-15:   MY4 blocks endothelial cell activation by LPS with and without serum. HUVEC in 96-well plates (5×10^4/well) were washed four times and then treated as indicated in the presence or absence of 10% serum (time, 6h; LPS, 1ng/ml; MY4, 1:25). E-selectin expression was measured by a two step ELISA. The data shown are the means ± SEM of sixtuplets of 3 independent experiments, each conducted with cells from a different cord.

8.2.3.11 Endothelial cell-associated CD14 has a functional role

To examine the functional relevance of CD14 expressed by HUVEC in terms of cell activation further, cultures were passaged for a total of seven times. When the MFI for CD14 had dropped to undetectable levels, these cells were grown to confluence in 96-well plates. After washing the plates three times, one half of the cells were kept in culture medium containing 10% serum, whereas the other half was kept in serum-free RPMI 1640 media. HUVEC were stimulated with either TNF (5U/well) or LPS (0.1-10ng/ml) for 6h, and E-selectin expression was assessed by ELISA. Only the cells kept in 10% serum responded to LPS. In contrast, TNF-α caused a similar up-regulation of E-selectin in these cells with and without serum present (Fig. 8-16A and 8-16B). In comparison, passage 1 HUVEC, (with normal expression of CD14), showed up-regulation of E-selectin in response to LPS in the absence of serum (Fig. 8-14C), albeit at a lesser magnitude than cells with serum (data not shown). This response was enhanced by the addition of serum (data not shown) and could be inhibited by the addition of MY4 (1:25) (Fig. 8-16C).
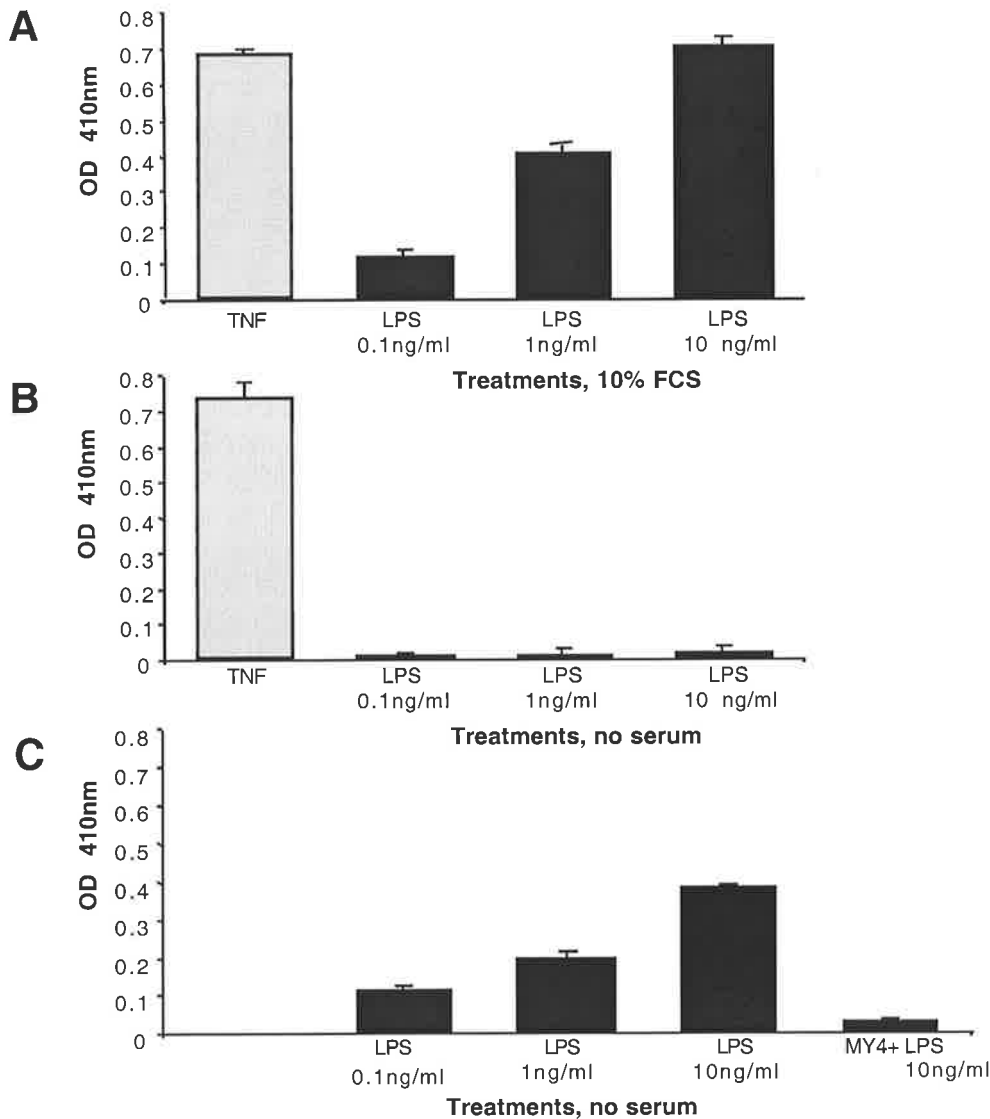
Figure 8-16: Activation of HUVEC by LPS via cell-associated CD14. As a measure of cell activation upregulation of E-selectin was assessed by ELISA after 6h of treatment. Results are the means ± SEM of quintuplets of 2 experiments with cells from 2 different cords and are expressed as optical density (OD) at 410nm. **A,B.** Passage 7 HUVEC, now lacking expression of CD14, were treated with TNF (5U/well) or LPS at the concentrations indicated. Cells were either treated in medium containing 10% serum or in serum-free conditions. **C.** Passage 1 HUVEC were treated with LPS in serum-free conditions. In addition, some cells were pre-treated with MY4 antibody (dilution 1:25) for 10min before LPS was added.

8.2.4   Respiratory Epithelial Cells

8.2.4.1 CD14 expression

In view of the observations by others (Striz et al., 1998) and the results described in chapter 6, (IFN-γ rendered A549 cells susceptible to LPS) it was examined whether A549 cells expressed CD14, and what influence IFN-γ might exert on CD14 expression. Encouraged by the discovery of CD14 on HUVEC, another cell type previously reported CD14-negative, the same staining technique was applied to A549 cells. When analyzed by flow cytometry there was a significant shift to the right of the

cell population treated with MY4 compared to cells treated with IgG_{2b} isotype-matched control antibody (Fig. 8-17).
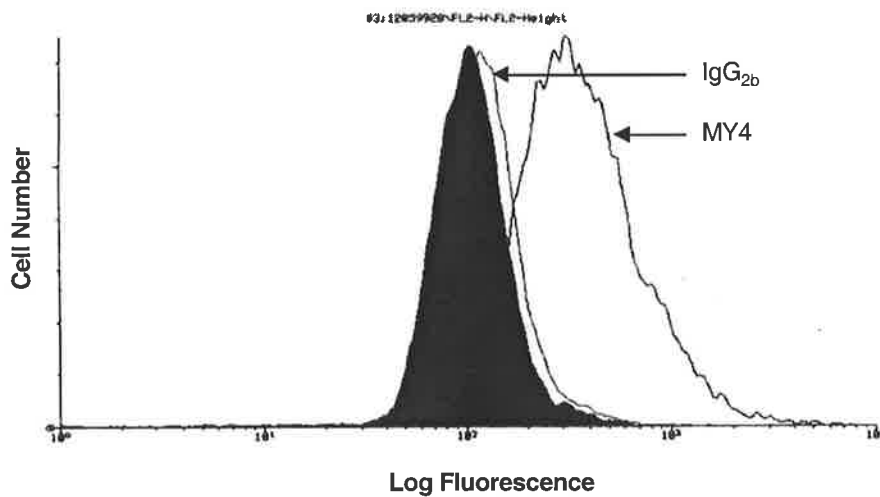
Figure 8-17:   CD14 expression on respiratory epithelial cells. A549 cells (10^6/assay) were enzymatically removed from culture dishes and either left unstained (auto-fluorescence, solid black, no antibodies) or stained with MY4 or IgG_{2b} isotype-matched control antibody as indicated by the arrows. The cells were washed and stained with R-PE-conjugated secondary antibody. A representative set of histogrammes is shown.

Interestingly, A549 cells displayed a high degree of auto-fluorescence (spontaneous fluorescence of an unstained cell population). The sensitivity of the detection method was improved with R-PE- compared to FITC-conjugated secondary antibody, and was higher again with the three-step staining technique (MY4 - biotinylated rat anti-mouse antibody - streptavidin - PE) (Fig. 8-18).
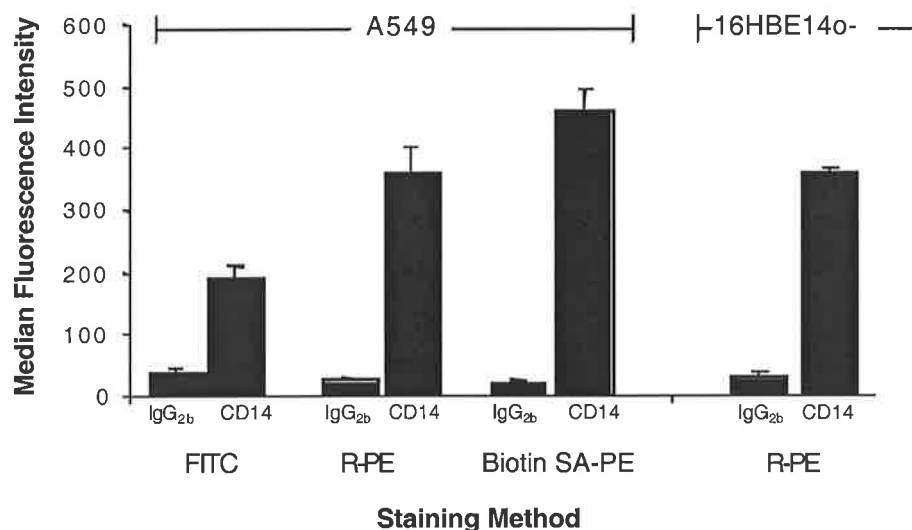
Figure 8-18:   CD14 expression on respiratory epithelial cells. Comparisons were made between the fluorescence intensities achieved in A549 cells using the 2-step indirect fluorescence method and FITC-conjugated secondary antibody, the two-step indirect fluorescence method and R-PE-conjugated secondary antibody and the three-step indirect fluorescence method (Mouse anti-human CD14 antibody, biotinylated rat anti-mouse antibody, SA-PE). Another human respiratory epithelial cell line, 16HBE14o-, was also stained for comparison (2-step R-PE method). The data are the means ± SEM of duplicates of 2 different experiments. For each method the results of isotype-matched control antibody (IgG_{2b}) and anti-CD14 antibody (MY4) are shown.

8.2.4.2 Estimation of the number of CD14 on respiratory epithelial cells

The level of expression of CD14 on the surface of A549 cells was estimated by comparison to monocytes and using FITC Quantum™ 26 beads as described in Fig. 8-11 (data not shown). The estimated number of CD14 per cell was found to be slightly higher than for HUVEC, $\sim$ 3,500 $\pm$ 500. The level of CD14 expression did not change when A549 cells were maintained in serum-free conditions for up to 48h (not shown). Another human immortilized respiratory epithelial cell line (16HBE14o-, Cozens et al., 1994), frequently used in cystic fibrosis research, was also tested for the expression of CD14. The level of expression was similar to that of A549 cells (Fig. 8-18).

8.2.4.3 Modulation of CD14 surface expression

It was examined next whether the expression of CD14 could be up- or downregulated in respiratory epithelial cells like observed in HUVEC, thus providing indirect evidence for synthesis of the CD14 molecule by this cell type. A549 cells were grown to confluence in 6-well plates and treated for 24h with either TNF-α (100U/ml), LPS (10μg/ml), IL-1β (1ng/ml), IFN-γ (1000U/ml), FCS (50%) or PMA (10nM). The expression of CD14 was moderately increased with TNF-α, IL-1β and IFN-γ, unchanged with LPS and FCS and downregulated slightly by PMA (Fig. 8-19A). Adding the protein synthesis inhibitor cycloheximide (CHX, 10μg/ml) for 24h resulted in a reduction of CD14 expression in untreated and LPS-treated cells with no alteration in cell viability as assessed by 7-AAD staining. CHX prevented the increase in CD14 seen with TNF-α and IL-1β (Fig. 8-19B).
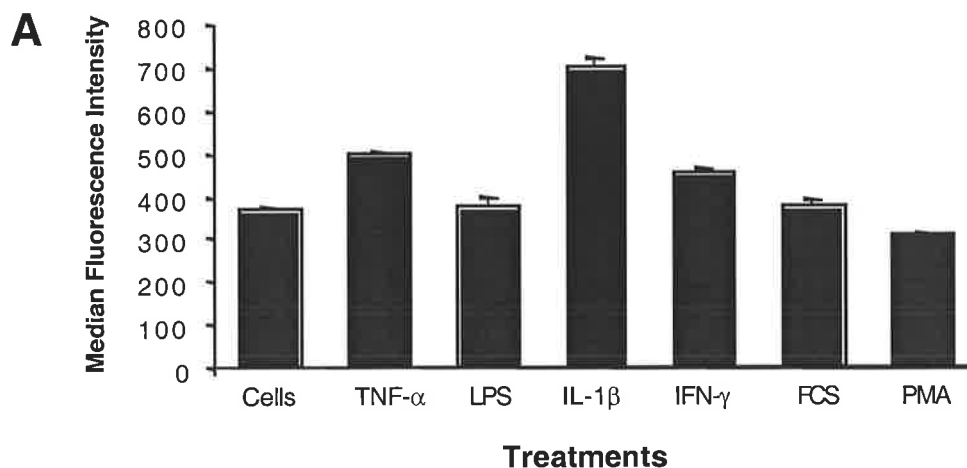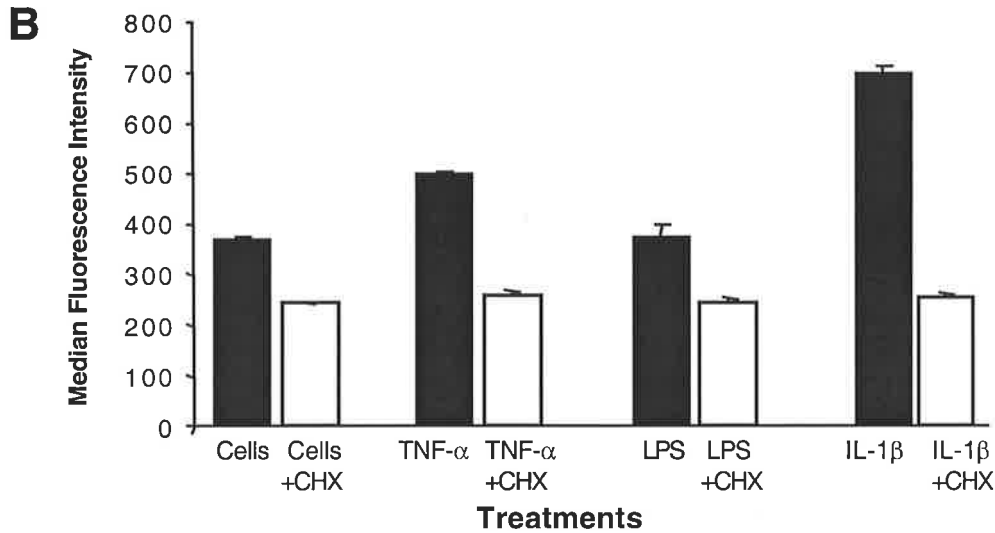
Figure 8-19: Modulation of CD14 surface expression in A549 cells. **A.** A549 cells (1×10^6/assay) were treated with the agonists indicated for 24h. TNF-α (100U/ml), +26%; LPS (10µg/ml), no change; IL-1β (1ng/ml), +47%; IFN-γ (1000U/ml), +19%; FCS (50%), no change; PMA (10nM), -12%. **B.** Cells were treated with either diluent, TNF-α, LPS or IL-1β in the absence or presence of CHX (10µg/ml). The expression of CD14 was assessed with indirect fluorescence and flow cytometric analysis of 10,000 viable cells/assay. The data are the means ± SEM of duplicates from 2 different experiments.

Results presented in chapter 6 showed that co-treatment with IFN-γ but not TNF-α or IL-1β rendered A549 cells responsive to LPS. When the time course of this interaction was studied it was established that pretreatment with IFN-γ for 2h produced a maximal effect. Thus the short-term effect of treatments on CD14 expression in A549 cells was examined. The cells were treated with TNF-α, LPS, IL-1β and IFN-γ (concentrations as above) for 1, 2 and 4 hours. There was a similar pattern of upregulation of CD14 as seen in the 24h experiment albeit to a lesser degree. There was no difference between the three time points studied, the results of the 4h experiment are shown (Fig. 8-20).
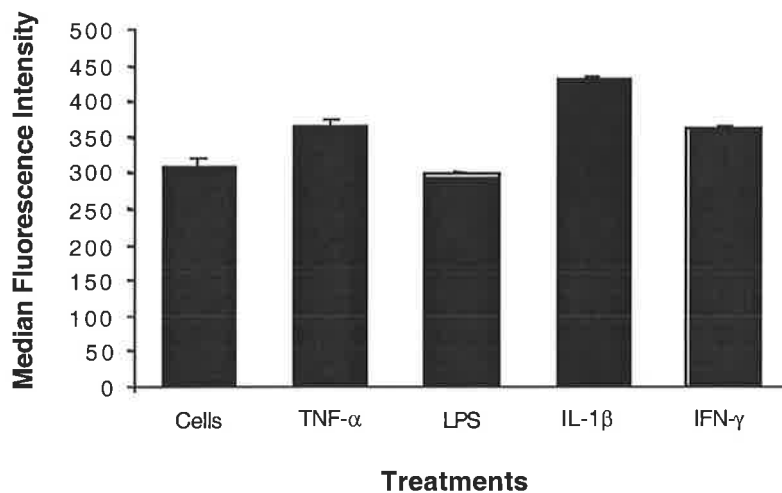
Figure 8-20: Effect of short-term treatment on CD14 expression in A549 cells. The cells were treated with the same concentrations of agonists as described in the legend of Fig. 8-20 for 4h. TNF-α (100U/ml), +16%; LPS (10µg/ml), no change; IL-1β (1ng/ml), +29%; IFN-γ (1000U/ml), +15.5%.

8.2.4.4   LPS-binding to Respiratory Epithelial Cells

The observation of LPS-binding being independent of CD14 in HBEC cells is intriguing (Striz et al., 1998). To determine whether this was also the case in alveolar type II cells (A549) confluent cells (10^6/assay) were removed from their culture dish and incubated on ice with either FITC-conjugated LPS or nonspecific FITC-conjugated IgG (mouse anti-human). Some cells were pre-incubated for 30 minutes with either IgG_{2b} or MY4 monoclonal antibody (dilution 1:10). The fluorescence intensity of the cell population was assessed by flow cytometric analysis of 10,000 events. There was significant binding of f-LPS to A549 cells, but surprisingly MY4 was unable to prevent binding of f-LPS (Fig. 8-21).
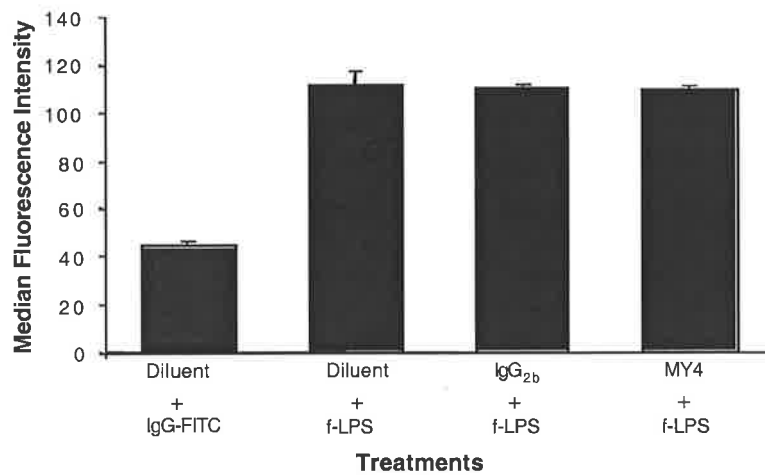
Figure 8-21:   Binding of LPS to respiratory epithelial cells. A549 cells (1×10^6/assay) were incubated on ice with either FITC-conjugated mouse IgG or FITC-conjugated LPS (f-LPS). Some cells were pre-incubated for 30min with either IgG_{2b} or MY4 (1:10) prior to exposure to f-LPS. The data represent the means ± SEM of duplicates of 3 independent experiments.

8.2.4.5   Search for other binding sites for LPS

MY4 being unable to prevent binding of LPS raises the question whether LPS is bound by different cell surface molecules (Table 1-5). A549 cells were screened for expression of the integrins CD11a, CD11b, CD11c, and CD18 with flow cytometric analysis and were found not to express any of these molecules on their surface (data not shown).

8.3   SUMMARY AND CONCLUSIONS

TNF-α treatment caused a significant increase in CD14 expression on neutrophils and an associated increase in LPS binding which could be blocked by an anti-CD14 monoclonal antibody. The magnitude of this increase correlated with the increase in the neutrophil chemiluminescence response as well as the adhesion to serum-coated plastic surfaces. Based on these observations, it is likely that TNF-α primes neutrophils for enhanced responses to LPS via an upregulation of surface CD14 leading to increased LPS binding.

Besides monocytes/macrophages and neutrophils both HUVEC and the respiratory epithelial cell line A549 express CD14 on their surface, albeit at substantially lower levels.

While previous studies have reported that human vascular endothelial cells lack the lipopolysaccharide (LPS) receptor, CD14, human vascular endothelial cells responded to LPS in serum-free conditions and this response could be abrogated by an anti-CD14 monoclonal antibody.

By optimizing assay conditions, it could be demonstrated that human umbilical vein endothelial cells express CD14 on the cell surface. Single passage HUVEC showed a density of ~2500 molecules per cell. Interestingly, there was significant loss of surface CD14 expression with increasing numbers of culture passages. Evidence for synthesis of CD14 by HUVEC was provided by the finding that ^{35}S L-methionine was incorporated into CD14. In addition, the expression of CD14 on HUVEC was up-regulated by LPS, lysophosphatidic acid (LPA) and tissue culture supplements and this up-regulation was dependent on protein synthesis. These results provide evidence for the first time that CD14 is expressed by endothelial cells and suggest that the previous inability to observe expression of this molecule has been due to culture and staining conditions.

Functional studies with the up-regulation of E-selectin as endpoint suggest that membrane-bound CD14 is essential for endothelial cell activation by LPS and that it acts in concert with serum factors (possibly soluble CD14).

We also demonstrated that A549 cells express CD14. Up- and down-regulation as well as dependence of CD14 expression on protein synthesis provided indirect evidence for CD14 being of epithelial cell origin. Furthermore, increasing the exogenous source of CD14 (sCD14 in serum in the culture medium) from 10% to 50% had no effect on the amount of CD14 on the cell surface. Although LPS bound to A549 cells, this interaction seemed to be independent of CD14, confirming the findings of Striz et al., 1998. This is an unusual observation and to our knowledge seems to be unique to epithelial cells. It is unlikely to be due to the fact that A549 cells are transformed, malignant cells, since CD14-independent binding of LPS had been shown in primary cell cultures (HBEC) as well (Striz et al., 1998).

Chapter 9

Discussion

9.1   REGULATION OF RESPIRATORY EPITHELIAL CELL ADHESION MOLECULE EXPRESSION BY MEDIATORS OF INFLAMMATION

ICAM-1 expression on type II pneumocytes can be induced following cell stimulation, and our data, derived from a respiratory type II epithelial cell line (A549), show that the cytokines TNF-α and IFN-γ rapidly upregulated ICAM-1in a synergistic manner. This suggests that this adhesion molecule is likely to participate in the regulation of the inflammatory reactions in the lung tissue, a finding in agreement with current published concepts (Tosi et al., 1992; Doerschuk et al., 1996). Apart from enhancing adhesion and migration of inflammatory cells to foci of inflammation ICAM-1 has also been shown to serve as a receptor for the Respiratory Syncytial Virus (RSV) in lung infection (Arnold et al., 1996). Synergism between TNF-α and IFN-γ has been shown to be relevant *in vitro* (Table 9-1) and *in vivo*. For example the combination of TNF-α and IFN-γ together with melphalan was successful in treating malignant tumours (Lienard et al., 1992).

Table 9-1: Synergism between TNF-α and IFN-γ

Cell type (All human)	Endpoint	Reference
Keratinocytes	Increased adhesion molecule expression and secretion of chemotactic factors	Barker et al., 1990
A549 cells	Increase in Mn-SOD	Harris et al., 1991
Melanoma cell line	Increased ICAM-1 expression	Jahnke et al., 1994
Aortic endothelial cells	Increase in FcγR expression	Pan et al., 1998
Neutrophils	Antibody-dependent cellular toxicity	Shalaby et al., 1995
Myeloid leukaemia cells (HL-60, ML3, U937)	NADPH oxidase activity	Cassatella et al., 1989
Endothelial cell line	NF-κB activation	Cheshire et al., 1997

A mechanism of how these cytokines may cooperate has not been found. While it has been described that IFN-γ enhances TNF receptor expression in six different transformed human cell lines (Tsujimoto et al., 1986) the number of TNF receptors was shown to be decreased in peripheral blood monocytes (Shepherd, 1991).

Treatment of A549 cells with a combination of LPS and TNF-α did not increase the expression of ICAM-1 significantly above the level seen with TNF-α alone. This is not surprising as LPS has been reported not to stimulate respiratory epithelial cells (Martin et al., 1994), a finding we confirmed in A549 cells with respect to ICAM-1 expression. Since LPS is found in significant amounts in the environment the insensitivity of the cells lining the airway to LPS is probably an adaptive response to prevent persistent and overwhelming inflammation in the lung (Martin et al., 1994). The lack of an increase in ICAM-1 expression in the presence of TNF-α and LPS could be

reproduced when experiments were repeated with IL-1β, another pyrogenic cytokine. Surprisingly, our results showed a synergistic increase in ICAM-1 with LPS and IFN-γ in A549 cells. It is not clear why IFN-γ but neither TNF-α nor IL-1β was able to synergize with LPS in A549 cells but this may be related to differences in the signalling mechanisms through which TNF-α and IL-1β activate cells compared to IFN-γ (Diagrams 1-11, 1-12 and 1-13).

The additive effect of TNF-α and IL-β on the up-regulation of ICAM-1 on the epithelial cell surface was not surprising. While adding IFN-γ to a combination of TNF-α and IL-β did not increase ICAM-1 expression beyond the level seen with TNF-α and IL-β only, ICAM-1 expression in A549 cells could be more than doubled by adding LPS to this 'cocktail' of cytokines (TNF-α, IL-1β and IFN-γ). This was remarkable given the absence of a synergistic effect between TNF-α and IL-β with LPS individually. The reason for this effect is not apparent, but such interaction has been reported previously with respect to an increase in iNOS in respiratory epithelial cells (Watkins et al., 1997).

The ability of cytokines to up-regulate the expression of ICAM-1 in respiratory cells suggests that TNF-α, IL-1β and IFN-γ promote lung inflammation. Our findings also establish that some mediators (e.g. IFN-γ) but not others (TNF-α, IL-1β) in combination with microbial products, such as LPS, significantly increase the expression of ICAM-1 on these cells. The observation that an already greatly enhanced cellular response to a cocktail of inflammatory cytokines could be more than doubled by LPS, which alone had no effect on ICAM-1 expression on epithelial cells, highlights how 'cross-talk' between mediators can alter the magnitude of an inflammatory reaction.

The respiratory epithelium is constantly exposed to microorganisms and microbial products prevalent in the environment. Studies with nasal, tracheal and alveolar epithelial cells have shown mostly similarities in their responses to stimuli, but also some differences. For the purpose of the work presented in this thesis an immortalized cell line (A549) was chosen as an *in vitro* model for respiratory epithelium. The reason for choosing a cell line was that primary human alveolar type II pneumocytes are not readily available and also that primary cells rapidly loose their characteristics in culture, displaying altered patterns of surface molecule expression and altered responses to stimuli. A549 cells, which are derived from a human adeno-carcinoma of the lung, have been well characterized and shown to be a valid model for studies of alveolar type II epithelial cells (Nardone et al., 1979).

9.2   EFFECTS OF TNF-α AND LPS ON VASCULAR ENDOTHELIAL CELL FUNCTION

The data presented in Chapter 5 demonstrate that TNF-α and LPS act synergistically on endothelial cells to increase the adhesive properties of this tissue. When added together at suboptimal concentrations, these mediators caused significantly greater expression of the adhesion molecules E-selectin, ICAM-1 and VCAM-1 than the sum of responses with either mediator alone. Interestingly, at

these concentrations the upregulation of endothelial adhesion molecules in response to TNF-α or LPS individually was not significantly different from the baseline. Because the induction of endothelial adhesion molecules is an important early step in the development of an inflammatory reaction, the synergism at very low concentrations of agonists described in this thesis is likely to be relevant in immunity to bacterial infections and associated pathology.

Endothelial adhesion molecules play an important role in regulating the movement of leukocytes from the blood to foci of inflammation (Fast et al., 1989; Frenette et al., 1996). The surface area of the pulmonary capillary bed alone is estimated to be $70m^2$ (Gee et al., 1993). When rats were challenged with immune complexes a prompt upregulation of the expression of E-selectin in the pulmonary vasculature was observed. This was associated with rapid neutrophil influx into the lung and vascular leakage. Pretreatment with anti-E-selectin antibody abolished both neutrophil influx and the damage to the vascular bed (Mulligan et al., 1991).

Upregulation of endothelial cell adhesion molecule expression increases leukocyte traffic across the endothelium to inflammatory foci. Although LPS-induced production of TNF-α significantly contributes to this process, LPS *per se* has the ability to induce or upregulate endothelial cell adhesion molecules directly (Beekhuizen et al., 1991; Schumann et al., 1996).

E-selectin and ICAM-1 are involved in the rapid recruitment of neutrophils, therefore their upregulation would influence acute infections with gram-negative organisms, such as pneumonia caused by *Haemophilus influenzae* or *Chlamydia pneumoniae*, cystitis by *E. coli* and meningitis by *Neisseria meningitides*. However, both E-selectin and ICAM-1 have been implicated in asthma, a chronic inflammatory condition (Wegner et al., 1990; Gundel et al., 1991). VCAM-1 is instrumental in recruiting monocytes, lymphocytes and eosinophils (Alon et al., 1995), hence its upregulation would be expected to be more important in chronic gram-negative infections, for example pulmonary colonization by *Pseudomonas aeruginosa* in cystic fibrosis patients or in chronic inflammation with eosinophil involvement such as seen in asthma. Induction of endothelial adhesion molecules is an important early step in the development of an inflammatory reaction (Mulligan et al, 1991; Qin et al., 1996), thus this synergism is likely to be relevant in immunity to bacteria.

Integrating the observation of synergistic up-regulation of endothelial adhesion molecules with the findings described in Chapter 3 and Chapter 4, it is evident how the vicious cycle of inflammation may operate. The LPS-induced cytokines produced by monocytes not only prime neutrophils but also act in concert with LPS to up-regulate endothelial adhesion molecules, thereby enhancing first adhesion and then migration of these primed neutrophils to foci of inflammation in tissues.

Synergism between LPS and IL-1β or other cytokines, or cytokines with cytokines with respect to neutrophil function was not studied. However, it is likely that similar patterns of responses will occur. Most of these pro-inflammatory cytokines are produced by macrophages in response to bacterial products and have overlapping effects on their target cells

9.3   THE INFLUENCE OF CYTOKINES ON LPS-INDUCED RESPONSES OF MONOCYTES AND MACROPHAGES

We found that LPS induced the production of TNF-α, IL-1β and IL-6 by monocytes and macrophages as previously established, but in addition our data showed that LPS synergized with TNF-α to markedly enhance the production of TNF-α and IL-1β. The LPS-induced production of IL-6 by monocytes was also enhanced by cytokines, but this effect was only modest in magnitude compared to the production of TNF-α and IL-1β. When examining these interactions we placed emphasis on conducting the experiments with low concentrations of LPS and of cytokines, as they are encountered during infections with gram-negative bacteria.

Mononuclear phagocytes are a major component of the innate immune system and found in abundance in lung tissue. Apart from releasing bactericidal substances, processing and presenting antigen, these cells secrete large quantities of TNF-α, IL-1β, and IL-6 in response to LPS (Haslett, 1987). We demonstrate here that these cytokines in turn are capable of greatly amplifying the effect of the bacterial product LPS to further influence production of proinflammatory mediators, thus constituting a quasi positive feedback loop. The resulting production of enhanced quantities of pro-inflammatory cytokines is likely to result in an enhanced activation of adjacent tissue cells, such as respiratory epithelium and vascular endothelium.

An example illustrating the complexity of this inflammatory network is provided by a recent study which demonstrated that human monocytes have an enhanced ability to eliminate intracellular bacteria when the cells are exposed to low doses of both LPS and cytokines (Kanangat et al., 1999). However, as the doses of LPS and the cytokine were increased the cells progressively lost this enhanced ability and finally the intracellular growth of bacteria increased again.

9.4   THE EFFECT OF TNF-α ON NEUTROPHIL RESPONSES TO LPS

Neutrophils preincubated with TNF-α for 20min showed a significantly enhanced production of oxygen-derived reactive species (ODRS) following LPS challenge. In contrast the response was only additive when the cells were treated with both mediators simultaneously. This suggests that TNF-α primes these leukocytes for increased responses to the bacterial product. This is consistent with previous observations that TNF-α primes neutrophils for increased killing of opsonized bacteria, fungi and parasites (Ferrante, 1989a; Ferrante, 1989b; Ferrante et al., 1993). When neutrophil adherence was examined it was found that the adhesion of the cells to serum-coated plastic surfaces was synergistically increased by treatment with LPS and TNF-α. This correlated with an increase in the surface expression of CD11b. We noted that the enhanced adhesion could be achieved with lower concentrations of agonists than were required for the upregulation of CD11b. This is in keeping with

observations by others and is likely to be explained by an increase in CD11b affinity rather than merely an increase in the level of cell surface expression. (Wright et al., 1991; Condliffe et al., 1996).

Neutrophils are the rapid deployment force of the innate immune system, within less than one hour after injecting animals with LPS more than 80% of the neutrophils were found in the lung (Haslett et al. 1987). Priming ensures that neutrophils have an enhanced ability to respond even to weak signals in infective foci (Yee et al., 1994). While our findings show such amplification of neutrophil responses in the presence of low concentrations of both TNF-α and LPS the implications of this effect *in vivo* remain to be studied.

Given the toxicity of the neutrophil-produced mediators, there often is collateral damage to local tissues, such as respiratory epithelial cells in a scenario of a pulmonary infection (Henson et al., 1987; Weiss, 1989; Ayars et al., 1994; Smith, 1994). This potentially detrimental role of neutrophils is also consistent with the observation that ARDS seldom occurs in neutropenic patients with sepsis (Sibille et al., 1990). Interestingly, studies in experimental murine neutropenia revealed that neutrophils have different roles in the defense against gram-positive or gram-negative bacteria. Granulocytopenic mice showed a severely impaired clearance of *Klebsiella pneumoniae,* whereas *Staphylococcus aureus* was cleared at a normal rate, the reason for this is not known. (Rehm et al., 1980).

9.5   MECHANISMS OF SYNERGISM BETWEEN TNF-α AND LPS

From previously published work it is evident that synergism or priming may be a result of increased expression of cell surface receptors and/or increased activation of intracellular signalling molecules. This was addressed in relation to synergism between LPS and TNF-α.

9.5.1   Changes in expression of CD14

There is controversy about whether respiratory epithelial cells express the LPS receptor CD14, perhaps arising from the fact that these cells are largely unresponsive to LPS (see also Chapter 8.1). Our data demonstrate that CD14 is present on the surface of respiratory epithelial cells and furthermore that CD14 is likely to be of epithelial cell origin.

Monocytes are the CD14-richest cells with about 50,000 copies per cell. These cells respond to picogram quantities of LPS. Neutrophils express CD14 weakly, about 10-fold less than monocytes. Interestingly, while endothelial cells are sensitive to low concentrations of LPS, no evidence has been presented to suggest that CD14 is expressed by these cells and indeed it has been generally accepted that endothelial cells do not express CD14 (Beekhuizen et al., 1991). In the absence of membrane-bound CD14 (mCD14), a soluble form of the antigen (sCD14), that is present in normal serum at 2-

6μg/ml, or the addition of serum is thought to facilitate LPS-induced cell activation (Frey et al., 1992). However, genetically CD14-negative cells like Chinese hamster ovary fibroblasts (CHO) and murine pre-B cells (70Z/3) are either unresponsive to LPS (CHO) or only respond to very high concentrations of LPS (70Z/3), even in the presence of serum. These cells become highly sensitive to LPS when transfected with CD14 (Lee et al., 1992; Golenbock et al., 1993). Surprisingly, anti-CD14 antibodies block endothelial cell activation by LPS even in the absence of serum (Von Asmuth et al., 1993). Both these observations are inconsistent with the concept that endothelial cells do not express CD14.

Given the importance of CD14 in LPS-mediated cell activation and the implications for the understanding of LPS-associated vascular disease it was decided to revisit the issue of CD14 expression by endothelial cells. By optimizing assay conditions, including the selection of anti-CD14 monoclonal antibody, it could be demonstrated that human umbilical vein endothelial cells (HUVEC) express CD14 on the cell surface. Single passage HUVEC showed a density of ~2500 molecules per cell, a relatively small number of molecules compared to monocytes. The CD14 molecules were of endothelial cell origin and not passively acquired from serum. Firstly, their surface expression was up- and down-regulated by various cell agonists. Secondly, the expression of CD14 molecules was inhibited by the protein synthesis inhibitor, cycloheximide. This inhibition was reversible under all conditions examined, except when cells were treated with LPS and CHX. LPS and CHX-treated cells displayed the same viability as control cells, grew in culture for an additional 72h without detachment from the culture dish or from each other and appeared morphologically normal. The reason for the inability of these cells to re-express CD14 is currently not clear. Thirdly, endothelial cells synthesized CD14 as shown by the incorporation of radio labelled amino acid into this molecule. Interestingly, while there was a significant upregulation of cell surface CD14 by adding FCS to the culture media, human serum had no effect on the expression of the molecule. ECGS, a bovine pituitary extract, was also capable of up-regulating CD14. Both FCS and ECGS were LPS-free and did not stimulate E-selectin expression in HUVEC. This suggests that FCS and ECGS may contain a bovine growth factor(s), which stimulates the synthesis of CD14 and seems to be absent from human serum.

Since the discovery of CD14 on HUVEC challenged current dogma, an explanation was sought for the discrepancy between our and previously published data. First the influence of culture conditions on the expression of CD14 was examined. Routine passaging of primary cultures of HUVEC or purchasing HUVEC from tissue culture laboratories at passages 3 to 5 is widely practiced. When cells were subjected to multiple passaging, these cells were indistinguishable from passage 1 HUVEC in a number of properties. The passaged cells displayed normal morphology and responded to TNF-α to the same extent as passage 1 cells. However, unlike passage 1 cells, HUVEC that had undergone multiple passaging expressed extremely low amounts of CD14. While the reason for this reduction in surface CD14 is not clear, it is likely that this reduction constitutes the main explanation for the previously reported lack of CD14 on the endothelial cell surface. The choice of monoclonal antibody against CD14 for the flow cytometric analysis may have been an additional limitation to the detection of CD14 on endothelial cells. Although comparisons between all available anti CD14

antibodies were not conducted, it is surprising how differently the antibodies tested performed, only MY4 and TÜK4 produced a positive stain in HUVEC.

Another distinction between passage 1 cells and cells that had undergone multiple passaging was the responsiveness to LPS in serum-free conditions. Cells subjected to several passages, which expressed very low amounts of CD14 failed to respond to LPS, but their responsiveness could be restored by the addition of serum. Passage 1 cells responded to LPS in the absence of serum in a CD14-dependent manner, and this response could be augmented by serum. These data demonstrate that, despite the low numbers of CD14 on HUVEC compared to monocytes, mCD14 on HUVEC is functional. Furthermore, mCD14 is required for the response of HUVEC to LPS in the absence of serum and acts in concert with serum factors in the presence of serum. Previous studies in macrophages and neutrophils showed that sCD14 facilitates the rapid transfer and efficient presentation of LPS to mCD14 (Hailman et al., 1994). This is likely to account for the augmentation of the LPS response in passage 1, mCD14-bearing endothelial cells. Taken together the data suggest that in LPS-induced activation of endothelial cells *in vivo* mCD14 is essential and sCD14 promotes the response by presenting LPS to mCD14. This concept is further supported by the transfection studies discussed above (Lee et al., 1992; Golenbock et al., 1993).

The pathophysiological importance of CD14 expressed on the endothelial cell surface would be significant. There is evidence that CD14 not only binds to LPS molecules but also to bacterial cell wall fragments and to whole bacteria (Jack et al., 1995; Noel et al., 1995; Katz et al., 1996). In addition to soluble LPS direct contacts between bacteria and endothelial cell-associated CD14 in bacteriaemia could thus result in an enhancement of the activation of this tissue. Activated endothelial cells upregulate adhesion molecules, secrete pro-inflammatory, pro-coagulatory mediators and vaso-active substances, such as eicosanoids, nitric oxide and endothelin, leading to disseminated intravascular coagulation (DIC), circulatory shock and organ failure (Vane et al., 1990).

A possible explanation for the synergistic effect in the upregulation of ICAM-1 expression in A549 cells seen with LPS and IFN-γ could have been an increase in expression of LPS receptor(s) on the surface of respiratory epithelial cells. However, we found that IFN-γ caused only a minimal increase in epithelial cell CD14 expression, and that there was no change in expression by treatment with LPS, TNF-α and IL-1β. Furthermore, the binding of LPS to epithelial cells was found to be CD14 independent, an observation also described by others (Striz et al., 1998). Thus, we do not know the significance of CD14 expression in respiratory epithelial cells. It is currently not known which structures on the epithelial cell surface are responsible for LPS binding. A549 cells do not express β-integrins, other known binding sites for LPS (Chapter 8.2.4.5). Human Toll-like receptors are likely candidates, although their expression on respiratory epithelial cells has not yet been reported. However, antibodies to hTLR-2 and hTLR-4 were not available at the time when the experiments for this thesis were carried out.

When the possibility was explored that the enhancement of LPS-induced cytokine production by mononuclear phagocytes was associated with changes in the level of expression of the LPS

receptor CD14 on the cell surface after exposure to cytokines, treatment of monocytes with TNF-α or LPS did not cause a change in CD14 expression in this cell type.

In contrast to the above the increase in neutrophil ODRS production was correlated with an increase in binding of LPS to the neutrophil surface and a parallel increase in CD14 expression. These observations and the time characteristics of the effect suggest that TNF-α primes neutrophils for enhanced LPS-induced ODRS production by rapidly translocating CD14 from its intracellular storage site, the azurophilic granules (Rodeberg et al., 1997), to the cell surface.

It has been recognized for some time that neutrophils can be primed by a variety of agents (Dahinden et al., 1983; Cochrane, 1987), but despite extensive research the mechanisms underlying this priming process are currently not known. The finding of CD14 upregulation as a potential mechanism seems inconsistent with the claim that TNF-α-induced release of the contents of azurophilic granules only occurs together with activation of the NADH-oxidase complex and therefore signifies comprehensive cell activation (Steadman et al., 1996). However, while the TNF-α used by this group was of the same origin as the TNF-α used for the work described in this thesis, the dose used by Steadman et al. was 200 fold higher than the doses we used to prime neutrophils. It is possible that at lower doses differential effects on cell function can be observed which are no longer discernible at very high concentrations of TNF-α.

Another theory as to how priming occurs is that the first agonist stimulates the intracellular activity of phospholipase A_2 (PLA_2), enabling the cell to show an enhanced response to a second mediator (Bauldry et al., 1991; Forehand et al., 1993). Interestingly, it has been shown that in the reverse to the experiments outlined in Chapter 4 neutrophils can be primed by LPS for enhanced responses to second agonists such as TNF-α (Guthrie et al., 1984; Shalaby et al., 1985; Aida et al., 1990). Others have demonstrated that the neutrophil respiratory burst is differentially regulated depending on the nature of the stimulus (Bellavite et al., 1992).

As outlined previously we showed for the first time that vascular endothelial cells express CD14. Although TNF-α synergizes with LPS in this cell type in terms of adhesion molecule expression, treatment of HUVEC with the cytokine did not alter the surface expression of CD14. In contrast LPS was able to up-regulate its receptor by about 60%.

9.5.2   Intracellular Signalling

Evidence has been presented that at least in endothelial cells the synergism between LPS and TNF-α operates at an intracellular signalling level. Since LPS and TNF-α were synergistic in expression of adhesion molecule mRNA, the effects of these mediators must be converging upstream of transcription.

The transcription factors activated in endothelial cell adhesion molecule expression are NF-κB and AP-1, the latter comprising of c-jun and ATF-2 (Manning et al., 1995; Brostjan et al., 1997). ATF-

2 knock-out mice show a blunted upregulation of endothelial E-selectin in response to stimuli (Reimold et al., 1996). In contrast, activation of c-jun and ATF-2 enhances NF-κB-induced E-selectin expression (Gupta et al., 1995). TNF-α and LPS are known to individually activate NF-κB in endothelial cells (Min et al., 1997). The data presented in Chapter 7 showed a synergistic increase in translocation of the amount of the Rel A (p65) sub-unit of NF-κB into the nucleus. Particularly interesting was the observation that in the presence of both TNF-α and LPS the normally transient activation of NF-κB persisted. This may be of clinical relevance, since persisting NF-κB activation is associated with amplification and perpetuation of the inflammatory response (Barnes et al., 1997) and also with the initiation and progression of atherosclerotic disease (Collins, 1993).

AP-1 and its two components, c-jun and ATF-2, were not measured directly, instead the activities of JNK and p38, the MAP kinases directly upstream of these transcription factors were assayed. TNF-α and LPS were synergistic in the upregulation of the activity of p38, the activity of JNK was increased in an additive manner.

It is generally accepted that the transcription factor Elk-1 does not play a major role in adhesion molecule gene activation. However, there have been reports to the contrary. This matter was investigated by assessing the activity of the Elk-1 MAP kinase, ERK, which, like in most adherent cell types, was significantly activated in unstimulated HUVEC. While both TNF-α and LPS individually caused a slight increase in the baseline activity of ERK, there was no enhancement with costimulation, which is consistent with the current concept of the role of ERK in the regulation of endothelial cell adhesion molecule expression (Read et al., 1997).

Taken together, our data suggest that NF-κB, ATF-2, and c-jun but not Elk-1 are likely to be the transcription factors targeted by the TNF-α and LPS-induced signals. We found the interactive effect between TNF-α and LPS to be most profound on NF-κB activation. This is consistent with the report by others, that although all three transcription factors are required for maximal adhesion molecule expression NF-κB plays the most important role (Brostjan et al., 1997). In this study glucocorticoid steroids did not inhibit the activation of c-jun or ATF-2, but blocked activation of NF-κB. This resulted in a near complete inhibition of E-selectin expression. This and similar observations led some investigators to propose that pharmacological inhibition of NF-κB may become the future basis for treatment of inflammation (Baeuerle et al., 1994; Chen et al., 1995).

A central question is where the point of convergence of TNF-α and LPS-initiated signalling pathways occurs. Based on published evidence it is most likely that both TNF-α and LPS lead to the activation of NF-κB via their signalling pathways joining downstream of TRAF2 and TRAF6 respectively (Diagram 1-14). NF-κB inducing kinase (NIK) is most probably this point of convergence (Cao et al., 1996b; Min et al., 1997; O'Neill et al., 1998; Pober, 1998).

The point of convergence for the JNK pathway are most likely the small GTP-binding proteins Rac and Cdc42. LPS activates these G-proteins via TRAF6, while TNF-α-mediated activation is through the Germinal Center Kinase and Germinal Center Kinase-Related via TRAF2 (see also

Diagrams 1-10,1-12 and 1-13). However, how the signals induced by TNF-α and LPS converge to activate p38 is currently not known. Based on the data presented in Chapter 7 and on published evidence it is unlikely that ceramide mimics LPS or plays a major role in the signalling events initiated by TNF-α and LPS, but it seems to function as a signal-enhancing factor.

Although the NF-κB and JNK pathways diverge downstream of TRAF and are regarded as separate (Liu et al., 1996), recent studies have demonstrated that there is 'cross-talk' between the MAP kinase cascades and NF-κB (Hirano et al., 1996; Lee et al., 1997; Read et al., 1997). Perhaps the strongest evidence points to MEKK1, the upstream kinase kinase kinase of the JNK/p38 cascade being linked to the activation of NF-κB (Table 9-2).

Table 9-2: Evidence supporting 'cross-talk' between MEKK1 and the NF-κB pathway

Observation	References
Overexpression of MEKK1 leads to increased phosphorylation of IκBα and translocation of NF-κB into the nucleus.	Hirano et al., 1996; Read et al., 1997
Recombinant MEKK1 is able to directly phosphorylate and activate IκB kinase *in vitro*.	Lee et al., 1997
A dominant-negative, kinase-dead mutant of MEKK1 is able to inhibit TNF-α-induced activation of a NF-κB reporter gene.	Lee et al., 1997

However, the effect of MEKK1 on the NF-κB pathway may not be a physiologically significant interaction, given that overexpression of kinase-inactive MEKK1 did not block TRAF2-mediated NF-κB activation, while JNK activity was completely suppressed (Liu et al., 1996). It would be interesting to know whether there is the reverse interaction between the NF-κB pathway and MEKK-1 or other signalling molecules of the JNK cascade. This has only been examined once, and in this study NIK was found not to be not involved in JNK activation (Song et al., 1997). In contrast, JNK has been shown to bind to the NF-κB constituent c-Rel and enhance transcription (Meyer et al., 1996).

Interactions between p38 and the NF-κB pathway have also been described. A recent report showed that inhibition of p38 alpha inhibits NF-κB, at least in neutrophils (Nick et al. 1999). In addition SB203580, a highly specific inhibitor of p38, prevents the expression of a NF-κB-controlled reporter construct in response to TNF-α stimulation as does insertion of a kinase-dead MKK6 mutant. However, affected is only the transactivation activity and not translocation into the nucleus or DNA binding, suggesting p38 may be required for maximal effect of NF-κB on gene transcription (Beyaert et al., 1996; Wesselborg et al., 1997).

Thus, it is possible that the combined effect of TNF-α and LPS on p38 and MEKK1 could lead to further amplification of NF-κB activation.

9.6 THE ROLE OF THE LPS RECEPTOR CD14 IN IMMUNITY AND DISEASE

As a pattern recognition receptor for bacterial products, CD14 plays an important part in innate immunity (Wright, 1995; Haziot et al., 1996; Schimke et al., 1998). Apart from the discovery of CD14 expression on vascular endothelial cells and respiratory epithelial cells presented in this thesis, the molecule has also been demonstrated to be present on cardiac myocytes. In this cell type CD14 is thought to be responsible for LPS-mediated cardio-depressant effects including LPS-induced myocyte apoptosis (Comstock et al., 1998).

Although the capacity of CD14 to signal has been disputed and numerous candidates for the LPS signalling receptor have been described, the functional significance of this molecule is striking. Blockade of CD14 with monoclonal antibody *in vitro* nearly completely abrogates LPS-mediated cell activation in both, mCD14-rich cells like monocytes and CD14-poor cells like neutrophils and vascular endothelial cells, regardless the presence of other LPS receptors such as hTLR-4.

Valuable information on the role of mCD14 can be gained from patients with paroxysmal nocturnal haemoglobinuria (PNH). These individuals cannot synthesize GPI anchors due to a genetic defect in the *PIG-A* gene, but are able to synthesize CD14. Monocytes of these patients express only very small amounts of mCD14. Despite an abundance of sCD14 in the serum of subjects with PNH their monocytes are 100- 1000-fold less sensitive to LPS (Duchow et al., 1993). This confirms the central role of mCD14 for LPS-mediated cell activation. In addition, the therapeutic administration of anti-CD14 antibody in animal models of septic shock is impressively successful (Matsuura et al., 1993; Leturcq et al., 1996; Schimke et al., 1998). In the latter trial anti-CD14 was beneficial even when administered after the induction of septic shock.

Genetically altered mice provide an excellent tool for dissecting the importance of various aspects of the action of LPS. CD14-deficient mice were 10,000 fold less sensitive to LPS and when infected with gram-negative bacteria had a reduced general inflammatory response, which was expected. That these mice also had a markedly reduced dissemination of bacteria compared to normal mice was surprising and is consistent with CD14 having a major role in the invasion of endothelial cells by bacteria (Haziot et al., 1996). Interestingly, LPS was able to induce acute-phase proteins in these CD14-deficient mice, suggesting that this effect of LPS may be mediated in a CD14-independent way (Haziot et al., 1998). To demonstrate that the induction of acute-phase proteins was definitely mediated by LPS, Haziot et al. repeated the experiments with CD14-positive, LPS gene-defective mice (C3H/HeJ). In contrast to CD14 deficient mice, LPS failed to induce acute-phase proteins in these animals.

The peripheral blood monocytes of LBP-deleted mice were 1,000-fold less sensitive to LPS than the cells of normal mice. But unexpectedly, when the LBP (-/-) animals were injected with LPS there were no differences in the responses of these animals compared to wild type mice. However, in

contrast to CD14 (-/-) mice, LBP (-/-) mice had a greatly increased bacterial dissemination when challenged with gram-negative bacteria (Wurfel et al., 1997; Fenton et al., 1998).

These observations lead to the following concept about the roles of LBP and CD14: LBP is a non-obligatory enhancer of LPS presentation to CD14 but may be required to opsonize gram-negative bacteria. mCD14 is required and sufficient for LPS-induced cell activation and is likely to facilitate the attachment of gram-negative organisms to vascular endothelial cells and their subsequent dissemination.

Although there are no reports of naturally occurring LBP (-/-) or CD14 (-/-) genotypes in humans, recently a girl with defective signalling by IL-1β and LPS was described (Kuhns et al., 1997). She presented with recurrent, severe bacterial infections, each with a reduced febrile response. IL-1β receptor and mCD14 expression were normal, LBP levels were not determined. The defect was deemed to be in the mutual signalling cascade initiated by LPS (via hTLR) and IL-1β.

Another recent discovery in humans emphasizes the role of CD14. A polymorphism in the CD14 promoter region was identified. This polymorphism was homozygous in 22% of 2541 people screened (Unkelbach et al., 1999; Hubaceck et al., 1999). When the homozygous individuals were divided into groups with coronary artery disease (CAD) and normals, the incidence was 27.5% and 15.6% respectively. This association of a CD14 polymorphism with CAD may be the pathophysiological basis for the observed link between gram-negative infections and atherosclerotic vascular events (Campbell et al., 1998; Kane et al., 1999; Schussheim et al., 1999). In an attempt to explain the mechanism for such association one group measured CD14 expression in peripheral blood monocytes and found that the level of CD14 expression on the cell surface was increased by 40% in individuals homozygous for the polymorphism (Hubacek et al., 1999). In the light of our discovery of CD14 on endothelial cells it would be very interesting to compare the level of CD14 expression on vascular endothelial cells of subjects homozygous for the polymorphism to that in normal subjects.

Also recently CD14 has been linked to the pathophysiology of gastritis, which is characterized by leukocyte infiltration of the gastric mucosa. LPS of *Helicobacter pylori* stimulates monocytes to release proinflammatory mediators such as the chemokine IL-8 (Bliss et al., 1998).

9.7   CONCLUDING REMARKS

Ubiquitous in the environment, LPS is a potentially lethal molecule, implicated in the pathophysiological processes of many infections. It also has been known for over 100 years that endotoxins have a beneficial role in the development and function of the normal human immune system and that they enhance the hosts overall resistance to bacterial infections and perhaps malignancies. Pretreatment of mice with small doses of LPS significantly improves the elimination of *E. coli* and survival of the animals (Vuopio-Varkila et al., 1987), and it also induces significant protection against the toxic effects of *Pseudomonas aeruginosa* Exotoxin A (Zehavi-Willner et al.,

1991). Likewise mucosal immunization with killed bacteria leads to a more rapid influx of neutrophils resulting in enhanced clearance of *H. influenzae* from the lung (Foxwell et al., 1998). Fragments of gram-negative bacterial cell walls have been discovered to persist inside macrophages for months after resolution of the infection, possibly functioning as a non-specific enhancer of the innate immune system (Ginsburg, 1988).

While it is evident that cytokines such as TNF-α and microbial products such as LPS co-exist during the course of an infection, we still know little of the contribution made by these mediators in the pathogenic host response with respect to tissues and cells being exposed to both types of molecules. Products released by macrophages stimulated with such agents as LPS play important roles in stimulating other phagocytes to kill microorganisms (Ferrante, 1991; Ferrante et al., 1992). Our work shows that LPS co-operates with TNF-α to enhance the microbial killing capacity of neutrophils. In fact, the *in vivo* observation of the LPS-induced enhancement of resistance to infections is likely to be a consequence of this type of synergism between endogenous and exogenous cell activators. This concept was extended to other cells and tissue types including monocytes and macrophages, vascular endothelial cells and respiratory epithelial cells. The synergistic responses shown in all these cell types are likely to influence the accumulation and activation of inflammatory leukocytes at foci of infection.

However, it is evident that where synergistic responses are less localized and involve multiple organs and tissues irreversible tissue damage, shock, organ failure and death may be precipitated. The concept generated in this thesis that cytokines render cells more responsive to LPS therefore also has the potential of bringing about harm to the host. For example activation of the mononuclear system and release of TNF-α in vivo via a parasite infection leads to LPS hypersensitivity (Ferrante et al., 1984).

In neutrophils, but not in macrophages, endothelial and epithelial cells, synergism between TNF-α and LPS was related to the ability of TNF-α to alter the expression of CD14 on the surface of cells. When searching for other mechanisms for synergistic responses by TNF-α and LPS we demonstrated that, at least in endothelial cells, they were associated with alterations in the magnitude of intracellular signals. In particular synergistic activation of the transcription factor NF-κB was observed. Although not implicated in the mechanism of the synergistic response between TNF-α and LPS in human vascular endothelial cells we discovered that CD14 is expressed by this tissue. This is a novel finding and given the importance of the CD14 molecule in gram-negative infections and in cardiovascular disease is likely to have an impact on the understanding and the management of those conditions.

While the work described in this thesis represents an important step towards studying and understanding the combined effects of different inflammatory mediators it marks only the beginning of a substantial body of research needed to unravel the complexity of the interactions at work in infections. Some of the areas which remained untouched by us because they were beyond the scope of this thesis shall be briefly summarized here: the effects of products of inflammatory leukocytes on

bacteria themselves are important to consider, for example it has been shown that even small amounts of leukocyte hydrolases increase the release of LPS from bacteria into the circulation (Ferne et al., 1978). Furthermore, the effects of multiple mediators released by inflammatory cells on cell surface structures of both leukocytes and tissues need to be taken into account. In particular PLA2 and ODRS lead to degradation of cell surface proteoglycans, hence synergizing in terms of cell lysis (Dan et al., 1996; Dishon et al., 1967; Ginsburg et al., 1993;

Staub et al., 1982). In relevance to the our work one has to ask what effects could these mediators and perhaps other enzymes have on the cell surface expression of CD14 or hTLRs ? Finally, it is well known that during infections in vivo acute phase proteins are upregulated, some of which are binding-proteins for LPS. Thus, it is conceivable that changes in serum levels of LBP and BPI may interfere with the availability and biological activity of LPS.

In addition to these challenges other future work may extend the concept of this thesis to additional cell types relevant to diseases of the lung, such as fibroblasts and airway smooth muscle cells. It would also be interesting to examine combined effects of different bacterial products, namely toxins of gram-positive organisms. Also, the roles of other proinflammatory cytokines such as IL-1b, IL-6 and IFN-g need to be examined in more detail. Other locally produced mediators such as ODRS, proteinases, phospholipases and arachidonic acid derivatives ought to be investigated as to their enhancing or inhibitory roles.

Bibliography

Abraham, E., Glauser, M.P., Butler, T., Garbino, J., Gelmont, D., Laterre, P.F., Kudsk, K., Bruining, H.A., Otto, C., Tobin, E., Zwingelstein, C., Lesslauer, W. and Leighton, A. 1997. P55 tumor necrosis factor receptor fusion protein in the treatment of patients with severe sepsis and septic shock. JAMA **277**:1531-1538.

Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakanishi, K. and Akira, S. 1998. Targeted disruption of the MyD88 gene results in loss of IL-1 and IL-18-mediated function. Immunity **9**:143-150.

Adcock, I.M. 1997. Transcription factors as activators of gene transcription: AP-1 and NF-κB. Monaldi Ach. Chest. Dis. **52**:178-186.

Aggarwal, B.B., Eessalu, T.E. and Hass, P.E. 1985. Characterization of receptors for human tumor necrosis factor and their regulation by γ-interferon. Nature **318**:665-667.

Aida, Y. and Papst, M.J. 1990. Priming of neutrophils by lipopolysaccharide for enhanced release of superoxide. Requirement for Plasma but not for Tumor Necrosis Factor-α. J. Immunol. **145**:3017-3025.

Albelda, S.M. 1991. Endothelial and epithelial cell adhesion molecules. Am. J. Respir. Cell Mol. Biol. **4**:195-203.

Alon, R., Kassner, P.D., Carr, M.W., Finger, E.B., Hemler, M.E. and Springer, T.A. 1995. The integrin VLA-4 supports tethering and rolling in flow on VCAM-1. J. Cell Biol. **128**:1243-1253.

Altman, L.C., Ayars, G.H., Baker, C. and Luchtel, D.L. 1993. Cytokines and eosinophil-derived cationic proteins upregulate intercellular adhesion molecule-1 on human nasal epithelial cells. J. Allergy Clin. Immunol. **92**:527-536.

Appelmelk, B.J., An, Y.Q., Geerts, M., Thijs, B.G., de Boer, H.A., MacLaren, D.M., de Graaff, J. and Nuijens, J.H. 1994. Lactoferrin is a lipid A-binding protein. Infect. Immun. **62**:2628-2632.

Aracil, F.M., Bosch, M.A. and Municio, A.M. 1985. Influence of *E. coli* lipopolysaccharide binding to rat alveolar type II cells on their functional properties. Mol. Cell. Biochem. **68**:59-66.

Arditi, M., Zhou, J., Dorio, R., Rong, G.W., Goyert, S.M. and Kim, K.S. 1993. Endotoxin-mediated endothelial cell injury and activation: role of soluble CD14. Infect. Immun. **61**:3149-3156.

Arditi, M., Zhou, J., Torres, M., Durden, D.L., Stins, M. and Kwang, S.K. 1995. Lipopolysaccharide stimulates the tyrosine phosphorylation of mitogen-activated protein kinases p44,

p42 and p41 in vascular endothelial cells in a soluble CD14-dependent manner. J. Immunol. **155**:3994-4003.

Arnold, R and König, W. 1996. ICAM-1 expression and low-molecular-weight G-protein activation of human bronchial epithelial cells (A549) infected with RSV. J. Leukoc. Biol. **60**:766-771.

Asano, K., Chee, C.B.E., Gaston, B., Lilly, C.M., Gerard, C., Drazen, J.M. and Stamler, J.S. 1994. Constitutive and inducible nitric oxide synthase gene expression, regulation and activity in human lung epithelial cells. Proc. Natl. Acad. Sci. USA **91**:10089-100093.

Ayars, G.H., Altman, L.C., Rosen, H. and Doyle, T. 1984. The injurious effect of neutrophils on pneumocytes in vitro. Am. Rev. Respir. Dis. **130**:964-973.

Babior, B.M., Kipnes, R.S. and Curnutte, J.T. 1972. The production by leukocytes of superoxide, a potential bactericidal agent. J. Clin. Invest. **52**:741-744.

Babior, B.M. 1999. NADPH oxidase: an update. Blood **93**:1464-1476.

Baeuerle, P.A. and Henkel, T. 1994. Function and activation of NF-κB in the immune system. Annu. Rev. Immunol. **12**:141-179.

Bagchi, A., Viscardi, R.M., Taciak, V., Ensor, J.E., McCrea, K.A. and Hasday, J.D. 1994. Increased activity of interleukin-6 but not tumor necrosis factor-α in lung lavage of premature infants is associated with the development of bronchopulmonary dysplasia. Pediatr. Res. **36**:244-252.

Bahrami, S., Schlag, G., Yao, Y.-M. and Redl, H. 1995. Significance of translocation/endotoxin in the development of systemic sepsis following trauma and/or haemorrhage. Prog. Clin. Biol. Res. **392**:197-208.

Baldwin, G, Alpert, G., Caputo, G.L., Baskin, M., Parsonnet, J., Gillis, Z.A., Thompson, C., Silber, G.R., and Fleisher, G.R. 1991. Effect of polymyxin B on experimental shock from meningococcal and *Escherichia coli* endotoxins. J. Infect. Dis. **164**:542-549.

Ballou, L.R., Laulederkind, S.J.F., Rosloniec, E.F. and Raghow, R. 1996. Ceramide signalling and the immune response. Biochim. Biophys. Acta **1301**:273-287.

Barber, S.A., Detore, G., McNally, R. and Vogel, S. 1996. Stimulation of the ceramide pathway partially mimics lipopolysaccharide-induced responses in murine peritoneal macrophages. Infect. Immun. **64**:3397-3400.

Barker, J.N.W.N., Sarma, V., Mitra, R.S., Dixit, V.M. and Nickoloff, B.J. 1990. Marked synergism between tumor necrosis factor-α and interferon-γ in regulation of keratinocyte-derived adhesion molecules and chemotactic factors. J. Clin. Invest. **85**:605-608.

Barnes, P.J. and Karin, M. 1997. Nuclear factor-κB-A pivotal transcription factor in chronic inflammatory diseases. N. Engl. J. Med. **336**:1066-1071.

Bauldry, S.A., Bass, D.A., Cousart, S.L. and McCall, C.E. 1991. Tumor necrosis factor α priming of phospholipase D in human neutrophils. J. Biol. Chem. **266**:4173-4179.

Bazil, V., Horejsi, V., Baudys, M., Kristofova, H., Strominger, J.L., Kostka, W. and Hilgert, I. 1986. Biochemical characterization of a soluable form of the 53-kDa monocyte surface antigen. Eur. J. Immunol. **16**:1583–1589.

Bazil, V. and Strominger, J.L. 1991. Shedding as a mechanism of down-modulation of CD14 on stimulated human monocytes. J. Immunol. **147**:1567-1574.

Beekhuizen, H., Blokland, I., Corsel van Tilburg, A.J., Koning, F. and van Furth, R. 1991. CD14 contributes to the adherence of human monocytes to cytokine-stimulated endothelial cells. J. Immunol. **147**:3761-3767.

Bellavite, P., Chirumbolo, S., Mansoldo, C., Gandini, G. and Dri, P. 1992. Simultaneous assay for oxidative metabolism and adhesion of human neutrophils: evidence for correlations and dissociations of the two responses. J. Leukoc. Biol. **51**:329-335.

Bennett, B.L., Lacson, R.G., Chen, C.C., Cruz, R., Wheeler, J.S., Kletzien, R.F., Tomasselli, A.G., Heinrikson, R.L. and Manning, A.M. 1996. Identification of signal-induced IκB-α kinases in human endothelial cells. J. Biol. Chem. **271**:19680-19688.

Bernard, A., Boumsell, L. and Hill, C. 1984. Joint report of the first international workshop on human leucocyte differentiation antigens by the investigators of the participating laboratories. In: Bernard, A., Boumsell, L., Dausset, J., Milstein, C., Schlossman, S.F. eds. Leucocyte Typing, Human Leucocyte Differentiation Antigens Detected by Monoclonal Antibodies. New York: Springer-Verlag; pp82-108.

Bernard, G.R., Wheeler, A.P., Russel, J.A., Schein, R., Summer, W.R., Steinberg, K.P., Fulkerson, W.J., Wright, P.E., Christman, B.W., Dupont, W.D., Higgins, S.B. and Swindell, B.B. 1997. The effects of ibuprofen on the physiology and survival of patients with sepsis. N. Engl. J. Med. **336**:912-918.

Berry, L., Andrew, M., Post, M., Ofosu, F. and O'Brodovich, H. 1991. A549 lung epithelial cells synthesize anticoagulant molecules on the cell surface and matrix and in conditioned media. Am. J. Respir. Cell Biol. **4**:338-346.

Beutler, B., Greenwald, D., Hulmes, J.D., Chang, M., Pan, Y.-C.E., Mathison, J., Ulevitch, R. and Cerami, A. 1985. Identity of tumour necrosis factor and the macrophage-secreted factor cachectin. Nature **316**:552-554.

Bevilacqua, M.P., Pober, J.S., Mendrick, D.L., Cotran, R.S. and Gimbrone, Jr., M.A. 1987. Identification of an inducible endothelial-leukocyte adhesion molecule. Proc. Natl. Acad. Sci. USA **84**:9238-9242.

Bevilacqua, M.P., Stengelin, S., Gimbrone, Jr., M.A. and Seed, B. 1989. Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. Science **243**:1160-1164.

Beyaert, R., Cuenda, A., Vanden Berghe, W., Plaisance, S., Lee, J.C., Haegeman, G., Cohen P. and Fiers, W. 1996. The p38/RK mitogen-activated protein kinase pathway regulates interleukin-6 synthesis in response to tumour necrosis factor. EMBO J. **15**:1914-1923.

Bittleman, D.B. and Casale, T.B. 1995. Interleukin-8 mediates interleukin-1α-induced neutrophil transcellular migration. Am. J. Respir. Cell Mol. Biol. **13**:323-329.

Blank, C., Luz, A., Bendigs, S, Erdmann, A., Wagner, H. and Heeg, K. 1997. Superantigen and endotoxin synergize in the induction of lethal shock. Eur. J. Immunol. **27**:825-833.

Bliss, Jr., C.M., Golenbock, D.T., Keates, S., Linevsky, J.K. and Kelly, C.P. 1998. *Helicobacter pylori* lipopolysaccharide binds to CD14 and stimulates release of interleukin-8, epithelial neutrophil-activating peptide 78 and monocyte chemotactic protein 1 by human monocytes. Infect. Immun. **66**:5357-5363.

Bosco, M.C., Espinoza-Delgado, I., Rowe, T.K., Malabarba, M.G., Longo, D.L. and Varesio, L. 1997. Functional role for the myeloid differentiation antigen CD14 in the activation of human monocytes by IL-2. J. Immunol. **159**:2922-2931.

Bourgoin, S., Plante, E., Gaudry, M., Naccache, P.H., Borgeat, P. and Poubelle, P.E. 1990. Involvement of a phospholipase D in the mechanism of action of granulocyte-macrophage colony-stimulating factor (GM-CSF): priming of human neutrophils in vitro with GM-CSF is associated with accumulation of phosphatidic acid and diradylglycerol. J. Exp. Med. **172**:767-777.

Borish, L., Rosenbaum, R., Albury, L. and Clark, S. 1989. Activation of neutrophils by recombinant interleukin 6. Cell. Immunol. **121**:280-289.

Boyle, Jr, E.M., Kovacich, J.C., Canty, Jr., T.G., Morgan, E.N., Chi, E., Verrier, E.D. and Pohlman, T.H. 1998. Inhibition of nuclear factor-κB nuclear localization reduces human E-selectin expression and the systemic inflammatory response. Circulation **98**:II-282-II-288.

Brandtzaeg, P., Kierulf, P., Gaustad, P., Skulberg, A., Bruun, J.N., Halvorsen, S. and Sorensen, E. 1989. Plasma endotoxin as a predictor of multiple organ failure and death in systemic meningococcal disease. J. Infect. Dis. **159**:195-204.

Brostjan, C., Anrather, J., Csizmadia, V., Natarjan, G. and Winkler, H. 1997. Glucocorticoids inhibit E-selectin expression by targeting NF-κB and not ATF/c-Jun. J. Immunol. **158**:3836-3844.

Brown, D. 1993. The tyrosine kinase connection: how GPI-anchored proteins activate cells. Curr. Opin. Immunol. **5**:349-354.

Brues, A.M. and Shear, M.J. 1944. Chemical treatment of tumors. X. Reactions of four patients with advanced malignant tumors to injection of a polysaccharide from *Serratia marcescens* culture filtrate. J. Natl. Cancer Inst. **5**:195-208.

Campbell, L.A., Kuo, C.-C. and Grayston, J.T. 1998. *Chlamydia pneumoniae* and cardiovascular disease. Emerg. Inf. Dis. **4**:571-579.

Cao, Z., Henzel, W.J. and Gao, X. 1996. IRAK: a kinase associated with the interleukin-1 receptor. Science **271**:1128-1131. (a)

Cao, Z., Xiong, J., Takeuchi, M., Kurama, T. and Goeddel, D. 1996. TRAF6 is a signal transducer for interleukin-1. Nature **383**:443-446. (b)

Carswell, E.A., Old, L.J., Kassell, R.L., Green, S., Fiore, N. and Williamson, B. 1975. An endotoxin-induced serum factor that causes necrosis of tumors. Proc. Natl. Acad. Sci. USA **72**:3666-3670.

Cassatella, M.A., Hartman, L., Perussia, B. and Trinchieri, G. 1989. Tumor necrosis factor and immune interferon synergistically induce cytochrome b$_{-245}$ heavy-chain gene expression and nicotinamide-adenine dinucleotide phosphate hydrogenase oxidase in human leukemic myeloid cells. J. Clin. Invest. **83**:1570-1579.

Chaudhary, P.M., Ferguson, C., Nguyen, V., Nguyen, Oanh, Massa, H.F., Eby, M., Jasmin, A., Trask, B.J., Hood, L. and Nelson, P.S. 1998. Cloning and characterization of two Toll/Interleukin-1

receptor-like genes TIL3 and TIL4: evidence for a multi-gene receptor family in humans. Blood **91**:4020-4027.

Cheers, C., Janas, M., Ramsay, A. and Ramshaw, I. 1999. Use of recombinant viruses to deliver cytokines influencing the course of experimental bacterial infection. Immunol. Cell Biol. **77**:324-330.

Chen, C.C. , Rosenbloom, C.L., Anderson, D.C. and Manning, A.M. 1995. Selective inhibition of E-selectin, vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 expression by inhibitors of IκB-α phosphorylation. J. Immunol. **155**:3538-3545.

Chi, E., Mehl, T, Nunn, D.and Lory, S. 1991. Interaction of *Pseudomonas aeruginosa* with A549 cells. Infect Immun. **59**:822-828.

Chini, B.A., Fiedler, M.A., Milligan, L., Hopkins, T. and Stark, J.M. 1998. Essential roles of NF-κB and C/EBP in the regulation of intercellular adhesion molecule-1 after respiratory syncytial virus infection of human respiratory epithelial cell cultures. J. Virol. **72**:1623-1626.

Christ, W. J., Asano, O., Robidoux, A.L.C., Perez, M., Wang, Y., Dubuc, G.R., Gavin, W.E., Hawkins, L.D., McGuiness, P.D., Mullarkey, M.A., Lewis, M.D., Kishi, Y., Kawata, T., Bristol, J.R., Rose, J.R., Rossignol, D.P., Kobayashi, S., Hishinuma, I., Kimura, A., Asakawa, N., Katavama, K. and Yamatsu, I. 1995. E5531, a pure endotoxin antagonist of high potency. Science **268**:80-83.

Cochrane, C.G. 1987. The enhancement of inflammatory injury. A. Rev. Respir. Dis. **136**:1-2.

Cohn, Z.A. and Morse, S.I. 1960. Functional and metabolic properties of polymorphnuclear leucocytes. J. Exp. Med. **111**:689-704.

Coley, W.B. 1891. Contribution to the knowledge of sarcoma. Read before the Surgical Section of the New York Academy of Medicine, pp. 199-220.

Collins, T. 1993. Endothelial nuclear factor-κB and the initiation of the atherosclerotic lesion. Lab. Invest. **68**:499-508.

Collins, T., Palmer, H.J., Whitley, M.Z., Neish, A.S. and Williams, A.J. 1993. A common theme in endothelial activation. Insights from the structural analysis of the genes for E-selectin and VCAM-1. Trends Cardiovasc. Med. **3**:92-97.

Comstock, K.L., Krown, K.A., Page, M.T., Martin, D., Ho, P., Pedraza, M., Castro, E.N., Nakajima, N., Glembotski, C.C., Quintana, P.J. and Sabbadini, R.A. 1998. LPS-induced TNF-

alpha release from and apoptosis in rat cardiomyocytes: obligatory role for CD14 in mediating the LPS response. J. Mol. Cell Cardiol. **30**:2761-2775.

Condliffe, A.M., Chilvers, E.R., Haslett, C. and Dransfield, I. 1996. Priming differentially regulates neutrophil adhesion molecule expression/function. Immunology **89**:105-111.

Corral, L.G., Muller, G.W., Moreira, A.L., Chen, Y., Wu, M., Stirling, D. and Kaplan, G. 1996. Selection of novel analogs of thalidomide with enhanced tumor necrosis factor alpha inhibitory activity. Mol. Med. **2**:506-515.

Cox, G.W., Mathieson, B.J., Giardina, S.L. and Varesio, L. 1990. Characterization of IL-2 receptor expression and function on murine macrophages. J. Immunol. **145**:1719-1726.

Cox, G., Gauldie, J. and Jordana, M. 1992. Bronchial epithelial cell-derived cytokines (G-CSF and GM-CSF) promote the survival of peripheral blood neutrophils *in vitro*. Am. J. Respir. Cell Biol. **7**:507-513.

Cox, G., Crossley, J. and Xing, Z. 1995. Macrophage engulfment of apoptotic neutrophils contributes to the resolution of acute pulmonary inflammation in vivo. Am. J. Resp. Cell Mol. Biol. **12**:232-237.

Cozens, A.L., Yezzi, M.J., Kunzelmann, K., Ohrui, T., Chin, L., Eng, K., Finkbeiner, W.E., Widdicombe, J.H. and Gruenert, D.C. 1994. CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. Am. J. Respir. Cell Mol. Biol. **10**:38-47.

Crestani, B., Cornillet, P., Dehoux, M., Rolland, C., Guenounou, M. and Aubier, M. 1994.Alveolar type II epithelial cells produce interleukin-6 in vitro and in vivo. J. Clin. Invest. **94**:731-740.

Crowley, M.T., Harmer, S.L. and DeFranco, A.L. 1996. Activation-induced association of a 145-kDa tyrosine-phosphorylated protein with Shc and Syk in B lymphoctes and macrophages. J. Biol. Chem. **271**:1145-1152.

Dahinden, C., Galanos, C. and Fehr, J. 1983. Granulocyte activation by endotoxin. J. Immunol. **130**:857-862.

Dahlberg, P.S., Acton, R.D., Battafarano, R.J., Uknis, M.E., Ratz, C.A., Johnston, J.W., Haseman, J.R., Gray, B.H. and Dunn, D.L. 1996. A novel endotoxin antagonist attenuates tumor necrosis factor-α secretion. J. Surg. Res. **63**:44-48.

Danner, R.L., Elin, R.J., Hosseini, J.M., Wesley, R.A., Reilly, J.M. and Parillo, J.E. 1991. Endotoxemia in human septic shock. Chest **99**:169-175.

Darnell, Jr., J.E., Kerr, I.M. and Stark, G.R. 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science **264**:1415-1421.

Dean, N.M., McKay, R., Condon, T.P. and Bennett, C.F. 1993. Inhibition of protein kinase C-α expression in human A549 cells by antisense oligonucleotides inhibits induction of intercellular adhesion molecule 1 (ICAM-1) mRNA by phorbol esters. J. Biol. Chem. **269**:16416-16424.

DeFranco, A.L., Crowley, M.T., Finn, A., Hambleton, J. and Weinstein, S.L. 1998. The role of tyrosine kinases and MAP kinases in LPS-induced signaling. Prog. Clin. Biol. Res. **397**:119-136.

Delude, R.L., Savedra, Jr., R., Zhao, H., Thieringer, R., Yamamoto, S., Fenton, M.J. and Golenbock, D.T. 1995. CD14 enhances cellular responses to endotoxin without imparting ligand-specific recognition. Proc. Natl. Acad. Sci. USA **92**:9288-9292.

Dentener, M.A., Von Asmuth, E.J., Francot, G.J., Marra, M.N. and Buurman, W.A. 1993. Antagonistic effects of lipopolysaccharide-binding protein and bactericidal/permeability-increasing protein on lipopolysaccharide-induced cytokine release by mononuclear phagocytes. Competition for binding to lipopolysaccharide. J. Immunol. **151**:4258-4265.

Devitt, A., Moffatt, O.D., Raykundalia, C., Capra, J.D., Simmons, D.L. and Gregory, C.D. 1998. Human CD14 mediates recognition and phagocytosis of apoptotic cells. Nature **392**:505-509.

Dhainaut, J.F., Tenaillon, A., Le Tulzo, Y., Schlemmer, B., Solet, J.P., Wolff, M., Holzapfel, L., Zeni, F., Dreyfuss, D., Mira, J.P. et al. 1994. Platelet-activating factor receptor antagonist BN 52021 in the treatment of severe sepsis: a randomized, double-blind, placebo-controlled, multicenter clinical trial. Crit. Care Med. **22**:1720-1728.

Diamond, G., Russell, J.P. and Bevins, C.L. 1996. Inducible expression of an antibiotic peptide gene in lipopolysaccharide-challenged tracheal epithelial cells. Proc. Natl. Acad. Sci. USA **93**:5156-5160.

Dobbs, L.G. 1990. Isolation and culture of alveolar type II cells. Am. J. Physiol.**258**:L134-L147.

Doelman, C.J.A. and Bast, A. 1990. Oxygen radicals in lung pathology. Free Rad. Biol. Med. **9**:381-400.

Doerschuk, C.M., Quinlan, W.M., Doyle, N.A., Bullard, D.C., Vestweber, D., Jones, M.L., Takei, F., Ward, P.A. and Beaudet, A.L. 1996. The role of P-selectin and ICAM-1 in acute lung injury as determined using blocking antibodies and mutant mice. J. Immunol. **157**:4609-4614.

Duchow, J., Marchant, A., Crusiaux, A., Husson, C., Alonso-Vega, C., De Groote, D., Neve, P. and Goldman, M. 1993. Impaired phagocyte responses to lipopolysaccharide in paroxysmal nocturnal hemoglobinuria. Infect. Immun. **61**:4280-4285.

Duncan, Jr., R.L. and Morrison, D.C. 1984. The fate of *E. coli* lipopolysaccharide after the uptake of *E. coli* by murine macrophages in vitro. J. Immunol. **132**:1416-1424.

Dusi, S., Della Bianca, V., Donini, M., Nadalini, K.A. and Rossi, F. 1996. Mechanisms of stimulation of the respiratory burst by TNF in nonadherent neutrophils. J. Immunol. **157**:4615-4623.

Dziarski, R. 1994. Cell-bound albumin is the 70-kDa peptidoglycan-, Lipopolysaccharide-, and Lipoteichoic Acid-binding protein on lymphocytes and macrophages. J. Biol. Chem. **269**: 20431–20436.

Eck, M.J. and Sprang, S.R. 1989. The structure of tumor necrosis factor-a at 2.6 Å resolution. J. Biol. Chem. **264**:17595-17605.

Elias, J.A., Zheng, T., Einarsson, O., Landry,M., Trow, T., Rebert, N. and Panuska, J. 1994. Epithelial interleukin-11. J. Biol. Chem. **269**:22261-22268.

Erger, R.A. and Casale, T. 1995. Interleukin-8 is a potent mediator of eosinophil chemotaxis through endothelium and epithelium. Am. J. Physiol. **268**:L117-L122.

Essani, N.A., McGuire, G.M., Manning, A.M. and Jaeschke, H. 1995. Differential induction of mRNA for ICAM-1 and selectins in hepatocytes, Kupffer cells and endothelial cells during endotoxaemia. Biochem. Biophys. Res. Commun. **211**:74-82.

Fadok, V.A., Voelker, D.R., Campbell, P.A., Cohen, J.J., Barton, D.L. and Henson, P.M. 1992. Exposure or phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J. Immunol. **148**:2207-2216.

Fast, D.J., Schlievert, P.M. and Nelson, R,D. 1989. Toxic shock syndrome-associated staphylococcal and streptococcal pyrogenic toxins are potent inducers of tumor necrosis factor production. Infect. Immun. **57**:291-294.

Fearns, C., Kravchenko, V.V., Ulevitch, R.J. and Loskutoff, D.J. 1995. Murine CD14 gene expression in vivo: extramyeloid synthesis and regulation by lipopolysaccharide. J. Exp. Med. **181**:857-866.

Fearns, C. and Loskutoff. D.J. 1997. Role of tumour necrosis factor alpha in induction of murine CD14 expression by lipopolysaccharide. Infect. Immun. **65**:4822-4831.

Fearon, D.T. and Locksley, R.M. 1996. The instructive role of innate immunity in the acquired immune response. Science **272**:50-54.

Fekade, D., Knox, K., Hussein, K., Melka, A., Lalloo, D., Coxon, R.E. and Warrell, D.A. 1996. Prevention of Jarish-Herxheimer reactions by treatment with antibodies against tumor necrosis factor α. N. Engl. J. Med. **335**:311-315.

Fenton, M.J. and Golenbock, D.T. 1998. LPS-binding proteins and receptors. J. Leukoc. Biol. **64**:25-32.

Ferrante, A. and Thong, Y.H. 1978. A rapid one-step procedure for purification of mononuclear and polymorphonuclear leukocytes from human blood using a modification of the Hypaque-Ficoll technique. J. Immunol. Methods. **24**:389-393.

Ferrante, A., Carter, R.F., Ferluga, J. and Allison, A.C. 1984. Lipopolysaccharide hyperreactivity of animals infected with *Trypanosoma lewisi* or *Trypanosoma musculi*. Infect. Immun. **46**:501-506.

Ferrante, A. 1989. Augmentation of the neutrophil response to Naegleria fowleri by tumour necrosis factor alpha. Infect. Immun. **57**:3110–3115. (a.)

Ferrante, A. 1989. Tumour necrosis factor alpha potentiates neutrophil antimicrobial activity: increased fungicidal activity against torulopsis glabrata and candida albicans and associated increases in oxygen radical production and lysosomal enzyme release. Infect. Immun. **57**:2115–2122..(b.)

Ferrante, A., Staugas, R.E.M., Rowan-Kelly, B., Bresatz, S., Kumaratilake, L.M., Rzepczyk, C. and Adolf, G.R. 1990. Production of tumor necrosis factor alpha and beta by human mononuclear leukocytes stimulated with mitogens, bacteria, and malarial parasites. Infect. Immun. **58**:3996-4003.

Ferrante, A. 1991. Activation of neutrophils by interleukins-1 and –2 and tumor necrosis factors. Immunol. Series. **5**:417-435.

Ferrante, A., Kowanko, I. and Bates, E. J. 1992. Mechanisms of host tissue damage by cytokine-activated neutrophils. pp. 499 - 521. In: Granulocyte responses to cytokines: Basic and clinical research. Edited by Coffey, R.G., Marcel Dekker, New York.

Ferrante, A., Martin, A.J., Bates, E.J., Goh, D.H.B., Harvey, D.P., Parsons, D., Rathjen, D., Russ, G. and Dayer, J.-M. 1993. Killing of *Staphylococcus aureus* by tumor necrosis factor α-activated neutrophils: the role of serum opsonins, integrin receptors, respiratory burst and degranulation. J. Immunol. **151**:4821-4828.

Ferrero, E. and Goyert, S.M. 1988. Nucleotide sequence of the gene encoding the monocyte differentiation antigen, CD14. Nucl. Acids Res. **16**:4173.

Ferrero, E., Hsieh, C.-L., Francke, U. and Goyert, S.M. 1990. CD14 is a member of the family of leucine-rich proteins and is encoded by a gene syntenic with multiple receptor genes. J. Immunol. **145**:331-336.

Fischer, Jr., C.J., Agosti, J.M., Opal, S.M., Lowry, S.F., Balk, R.A., Sadoff, J.C., Abraham, E., Schein, R.M.H. and Benjamin, E. 1996. Treatment of septic shock with the tumor necrosis factor receptor:Fc fusion protein. N. Engl. J. Med. **334**:1697-1702.

Fisher, C.J.J., Dhainaut, J.F., Opal, S.M., Pribble, J.P., Balk, R.A., Slotman, G.J., Iberti, T.J., Rackow, E.C., Shapiro, M.J., Greenman, R.L., Reines, H.D., Shelly, M.P., Thompson, B.W., LaBrecque, J.F., Catalano, M.A., Knaus, W.A. and Sadoff, J.C. 1994. Recombinant human interleukin-1 receptor antagonist in the treatment of patients with sepsis syndrome: results from a randomized, double blind, placebo-controlled trial. JAMA **271**:1836-1843.

Fischer, G., Majdic, O., Gadd, S. and Knapp, W. 1990. Signal transduction in lymphocytic and myeloid cells via CD24, a new member of phosphoinositol-anchored membrane molecules. J. Immunol. **144**:638–641.

Forehand, J.R., Johnston, Jr., R.B. and Bomalaski, J.S. 1993. Stimulation by lipopolysaccharide and possible involvement in priming for an enhanced respiratory burst. J. Immunol. **151**:4918-4925.

Foxwell, R., Kyd, J.M. and Cripps, A.W. 1998. Characteristics of the immunological response in the clearance of non-typable *Haemophilus influenzae* from the lung. Immunol. Cell Biol. **76**:323-331.

Francis, G. 1999. TNF-α and heart failure. Circulation **99**:3213-3214.

Frenette, P.S. and Wagner, D.D. 1996. Adhesion molecules – Part II: Blood vessels and blood cells. N. Eng. J. Med. **335**:43-45.

Freudenberg, M.A., Kleine, B. and Galanos, C. 1984. The fate of lipopolysaccharide in rats: evidence for chemical alteration in the molecule. Rev. Infect. Dis. **6**:483-487.

Frey, E.A., Miller, D.S., Gullstein Jahr, T., Sundan, A., Bazil, V., Espevik, T., Finlay, B.B. and Wright, S.D. 1992. Soluble CD14 participates in the response of cells to lipopolysaccharide . J. Exp. Med. **176**: 1665-1671.

Frost, J.A., Geppert, T.D., Cobb, M.H. and Feramisco, J.R. 1994. A requirement for extracellular signal-regulated kinase (ERK) function in the activation of AP-1 by Ha-Ras, phorbol 12-myristate 13-acetate, and serum. Proc. Natl. Acad. Sci. USA **91**:3844-3848.

Fukahori, M., Murata, T., Mohammed, M.U., Fukuyama, N. and Nakazawa, H. 1997. Early reperfusion induces alveolar injury in pulmonary embolism. Chest **111**:198-203.

Gallay, P., Heumann, D., Le Roy, D., Barras, C. and Glauser, M.P. 1993. lipopolysaccharide-binding protein as a major plasma protein responsible for endotoxaemic shock. Proc. Natl. Acad. Sci. USA **90**:9935-9938.

Gamble, J.R., Smith, W.B. and Vadas, M.A. 1992. In: **Beutler, B.** (Ed.). Tumor Necrosis Factors, the molecules and their emerging role in medicine. Raven Press, New York, pp 65-86.

Gay, N.J. and Keith, F.J. 1991. *Drosophila* Toll and IL-1 receptor. Nature **351**:355-356.
Hahon, N. and Castranova, V. 1989. Interferon production in rat type II pneumocytes and alveolar macrophages. Exp. Lung Res. **15**:429-445.

Gee, M.H. and Albertine, K.H. 1993. Neutrophil-endothelial cell interactions in the lung. Annu. Rev. Physiol. **55**:227-248.

Gegner, J.A., Ulevitch, R.J. and Tobias, P.S. 1995. Lipopolysaccharide (LPS) signal transduction and clearance. Dual roles for LPS binding protein and membrane CD14. J. Biol. Chem. **270**:5320-5325.

Geng, Y., Gulbins, E., Altman, A. and Lotz, M. 1994. Monocyte deactivation by interleukin 10 via inhibition of tyrosine kinase activity and the Ras signaling pathway. Proc. Natl. Acad. Sci. USA **91**:8602-8606.

Geppert, T.D., Whitehurst, C.E., Thompson, P. and Beutler, B. 1994. Lipopolysaccharide signals activation of tumor necrosis factor biosynthesis through the ras/raf-1/MEK/MAPK pathway. Mol. Med. **1**:93-103.

Ginsburg, I. 1988. The biochemistry of bacteriolysis: paradoxes, facts and myths. Microbiol. Sci. **5**:137-142.

Giroir, B.P., Quint, P.A., Barton, P., Kirsch, E.A., Kitchen, L., Goldstein, B., Nelson, B.J., Wedel, N.I., Carroll, S.F. and Scannon, P.J. 1997. Preliminary evaluation of recombinant amino-terminal fragment of human bactericidal/permeability-increasing protein in children with severe meningococcal sepsis. Lancet **350**:1439-1443.

Glauser, M.P. 1996. The infammatory cytokines: new developments in the pathophysiology and treatment of septic shock. Drugs **52** (Suppl.2):9-17.

Goerdt, S. and Orfanos, C.E. 1999. Other functions, other genes: alternative activation of antigen-presenting cells. Immunity **10**:137-142.

Golenbock, D.T., Liu, Y., Millham, F.H., Freeman, M.W. and Zoeller, R.A. 1993. Surface expression of human CD14 in Chinese hamster ovary fibroblasts imparts macrophage-like responsiveness to bacterial endotoxin. J. Biol. Chem. **268**:22055-22059.

Goyert, S.M., Ferrero, E., Rettig, W.J., Yenamandra, A.K., Obata, F. and Le Beau, M.M. 1988. The CD14 monocyte differentiation antigen maps to a region encoding growth factors and receptors. Science **239**:497-500.

Goyert, S.M. and Haziot, A 1995. Recombinant soluble CD14 inhibits LPS-induced mortality in a murine model. Prog. Clin. Biol. Res. **392**:479-483.

Greenman, R.L., Schein, R.M.H., Martin, M.A., Wenzel, R.P., MacIntyre, N.R., Emmanuel, G., Chmel, H., Kohler, R.B., McCarthy, M., Plouffe, J. and Russel, J.A. 1991. A controlled clinical trial of E5 murine monoclonal IgM antibody to endotoxin in the treatment of gram-negative sepsis. JAMA **266**:1097-1102.

Griffin, J.D., Ritz, J., Nadler, L.M. and Schlossman, S.F. 1981. Expression of myeloid differentiation antigens on normal and myeloid cells. J. Clin. Invest. **68**:932-941.

Grigg, J.M., Savill, J.S., Sarraf, C., Haslett, C. and Silverman, M. 1991. Neutrophil apoptosis and clearance from neonatal lungs. Lancet **338**:720-722.

Grünwald, U., Krueger, C. and Schütt, C. 1993. Endotoxin-neutralizing capacity of soluble CD14 is a highly conserved specific function. Circulatory Shock **39**:220-225.

Guerrini, L., Casalino, L., Corti, A. and Blasi, F. 1996. NF-κB-mediated regulation of urokinase gene expression by PMA and TNF-α in human A549 cells. FEBS letters **393**:69-73.

Gundel, R.H., Wegner, C.D., Torcellini, C.A., Clarke, C.C., Haynes, N., Rothlein, R., Smith, C.W. and Letts, L.G. 1991. Endothelial leukocyte adhesion molecule-1 mediates antigen-induced acute airway inflammation and late-phase airway obstruction in monkeys. J. Clin. Invest. **88**:1407-1411.

Gupta, S., Campbell, D., Dérijard, B. and Davis, R.J. 1995. Transcription factor ATF2 regulation by the JNK signal transduction pathway. Science **267**:389-393.

Guthridge, C.J., Eidlen, D., Arend, W.P., Gutierrez-Hartmann, A. and Smith, M.F., Jr. 1997. Lipopolysaccharide and Raf-1 kinase regulate secretory interleukin-1 receptor antagonist gene expression by mutually antagonistic mechanisms. Mol. Cell. Biol. **17**:1118-1128.

Guthrie, L.A., McPhail, L.C., Henson, P.M. and Johnston, R.B., Jr. 1984. Priming or neutrophils for enhanced release of oxygen metabolites by bacterial lipopolysaccharide. J. Exp. Med. **160**:1656-1671.

Gyllenhammar, H. 1986. Lucigenin chemiluminescence in the assessment of neutrophil superoxide production. J. Immunol. Methods. **97**:209-213.

Hailman, E., Lichenstein, H.S., Wurfel, M.M., Miller, D.S., Johnson, D.A., Kelley, M., Busse, L.A., Zukowski, M.M. and Wright, S.D. 1994. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. J. Exp. Med. **179**:269-277.

Hall, A. 1994. A biochemical function for Ras – at last. Science **264**:1413-1414.

Hambleton, J., McMahon, M. and DeFranco, A.L. 1995. Activation of Raf-1 and mitogen-activated protein kinase in murine macrophages partially mimics lipopolysaccharide-induced signaling events. J. Exp. Med. **182**:147-154.

Hampton, R.H., Golenbock, D.T., Penman, M., Krieger, M. and Raetz, C.R.H. 1991. Recognition and plasma clearance of endotoxin by scavenger receptors. Nature **352**:342-344.

Han, J., Lee, J.-D., Tobias, P.S. and Ulevitch, R.J. 1993. Endotoxin induces rapid protein tyrosine phosphorylation in 70Z/3 cells expressing CD14. J. Biol. Chem. **268**:25009-25014.

Han, J., Lee, J.-D., Bibbs, L. and Ulevitch, R.J. 1994. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science **265**:808-811.

Hancock, J.T. 1997. Superoxide, hydrogen peroxide and nitric oxide as signalling molecules: their production and role in disease. Br. J. Biomed. Sci. **54**:38-46.

Harder, T., Scheiffele, P, Verkade, P. and Simons, K. 1998. Lipid domain structure of the plasma membrane revealed by patching of membrane components. J. Cell Biol. **141**:929-942.

Harder, T and Simons, K. 1999. Clusters of glycolipid and glycosylphosphatidylinositol-anchored proteins in lymphoid cells: accumulation of actin regulated by local tyrosine phosphorylation. Eur. J. Immunol. **29**:556-562.

Hardy, S.J., Ferrante, A., Poulos, A., Robinson, B.S., Johnson, D.W. and Murray, A.W. 1994. Effect of exogenous fatty acids with greater than 22 carbon atoms (very long chain fatty acids) on superoxide production by human neutrophils. J. Immunol. **153**:1754–1761.

Harris, C.A., Derbin, K.S., Hunte-McDonough, B., Krauss, M.R., Chen, K.T., Smith, D.M. and Epstein, L.B. 1991. Manganese superoxide dismutase is induced by IFN-γ in multiple cell types. J. Immunol. **147**:149-154.

Haslett, C., Worthen, G.S., Giclas, P.C., Morrison, D.C, Henson, J.E. and Henson, P.M. 1987. The pulmonary vascular sequestration of neutrophils in endotoxemia is initiated by an effect of endotoxin on the neutrophil in the rabbit. Am. Rev. Respir. Dis. **136**:9-18.

Haziot, A., Chen, S., Ferrero, E., Low, M.G., Silber, R. and Goyert, S.M. 1988. The monocyte differentiation antigen, CD14, is anchored to the cell membrane by a phosphatidylinositol linkage. J. Immunol. **141**:547-552.

Haziot, A., Rong, G.-W., Silver, J. and Goyert, S.M. 1993. Recombinant soluble CD14 mediates the activation of endothelial cells by lipopolysaccharide. J. Immunol. **151**:1500-1507.

Haziot, A., Rong, G.W., Lin, X.Y., Silver, J. and Goyert, S.M. 1995. Recombinant soluble CD14 prevents mortality in mice treated with endotoxin (lipopolysaccharide). J. Immunol. **154**:6529-6532.

Haziot, A., Ferrero, E, Köntgen, F., Hijiya, N., Yamamoto, S., Silver, J., Stewart, C.L. and Goyert, S.M. 1996. Resistance to endotoxin shock and reduced dissemination of gram-negative bacteria in CD14-deficient mice. Immunity **4**:407-414.

Heidenreich, S., Schmidt, M., August, C., Cullen, P., Rademaekers, A. and Pauels, H.-G. 1997. Regulation of human monocyte apoptosis by the CD14 molecule. J. Immunol. **159**:3178-3188.

Henderson, B. and Wilson, M. 1995. Modulins: a new class of cytokine-inducing, pro-inflammatory bacterial virulence factor. Inflamm. Res. **44**:187-197.

Henderson, B., Poole, S. and Wilson, M. 1996. Bacterial modulins: a novel class of virulence factors which cause host tissuepathology by inducing cytokine synthesis. Microbiol. Rev. **60**:316-341.

Henderson, B., Poole, S. and Wilson, M. 1996. Microbial/host interactions in health and disease: who controls the cytokine network? Immunopharmacol. **35**:1-21. (a)

Henderson, B and Wilson, M. 1996. Cytokine induction by bacteria: beyond lipopolysaccharide. Cytokine **8**:269-282. (b)

Hennet, T., Richter, C. and Peterhans, E. 1993. Tumour necrosis factor-α induces superoxide anion generation in mitochondria of L929 cells. Biochem. J. **289**:587–592.

Henson, P.M. and Johnston, Jr., R.B. 1987. Tissue injury in inflammation. J. Clin. Invest. **79**:669-674.

Hii, C.S.T., Huang, Z.H., Bilney, A., Costabile, M., Murray, A.W., Rathjen, D.A., Der, C.J. and Ferrante, A. 1998. Stimulation of p38 phosphorylation and activity by arachidonic acid in HeLa cells, HL60 promyelocytic leukaemic cells, and human neutrophils. J. Biol. Chem. **273**:19277-19282.

Hirano, M., Osada, S., Aoki, T., Hirai, S., Hosaka, M., Inoe, J. and Ohno, S. 1996. MEK kinase is involved in tumor necrosis factor α-induced NF-κB activation and degradation of IκB-α. J. Biol. Chem. **271**:13234-13238.

Hitchcock, P.J., Leive, L., Makela, P.H., Rietschel, E.T., Strittmatter, W. and Morrison, D.C. 1986. Lipopolysaccharide nomenclature-past, present and future. J. Bacteriol. **166**:699-705.

Hohmann, H.-P., Brockhaus, M., Baeuerle, P.A., Remy, R., Kolbeck, R and van Loon, A.P.G.M. 1990. Expression of the types A and B tumor necrosis factor (TNF) receptors is independently regulated, and both receptors mediate activation of the transcription factor NF-κB. J. Biol. Chem. **265**:22409-22417.

Holland, P.M., Suzanne, M., Campbell, J.S., Noselli, S. and Cooper, J.A. 1997. MKK7 is a stress-activated mitogen-activated protein kinase kinase functionally related to *hemipterous*. J. Biol. Chem. **272**:24994-24998.

Horwitz, A. 1997. Integrins and Health. Sci. Am. **276**:46-53.

Hubacek, J.A., Pit'ha, J., Skodová, Z., Stanek, V. and Poledne, R. 1999. C (-260) → T polymorphism in the promoter of the CD14 monocyte receptor gene as a risk factor for myocardial infarction. Circulation **99**:3218-3220.

Huber, A.R., Kunkel, S.L., Todd, III,R.F. and Weiss, S.J. 1991. Regulation of transendothelial neutrophil migration by endogenous interleukin-8. Science **254**:99-104.

Huizinga, T.W.J., van der Scoot, C.E., Jost, C., Klaassen, R., Kleijer, M., von dem Borne, A.E.G.Kr., Roos, D. and Tetteroo, P.A.T. 1988. The PI-linked receptor FcRIII is released on stimulation of neutrophils. Nature **333**:667-669.

Hussain, S., Zwilling, B.S. and Lafuse, W.P. 1999. Mycobacterium avium infection of mouse macrophages inhibits IFN-γ janus kinase-STAT signaling and gene induction by down-regulation of the IFN-γ receptor. J. Immunol. **163**:2041-2048.

Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K. and Gotoh, Y. 1997. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science **275**:90-94.

Ikeda, A., Takata, T., Taniguchi, T. and Sekikawa, K. 1997. Molecular cloning of bovine CD14 gene. J. Vet. Med. Sci. **59**:715-719.

Ingalls, R.R. and Golenbock, D.T. 1995. CD11c/CD18, a transmembrane signaling receptor for lipopolysaccharide. J. Exp. Med. **181**:1473-1479.

Ingalls, R.R., Arnaout, M.A., Delude, R.L., Flaherty, S., Savedra, Jr., R. and Golenbock, D.T. 1998. The CD11/CD18 integrins: characterization of three novel LPS signaling receptors. Prog. Clin. Biol. Res. **397**:107-117.

Jack, R.S., Grünwald, U., Stelter, F., Workelamahu, G. and Schütt, C. 1995. Both membrane-bound and soluble forms of CD14 bind to gram-negative bacteria. Eur. J. Immunol. **25**:1436-1441.

Jaffe, E.A., Nachman, R.L., Becker, C.G. and Minick, C.R. 1973. Culture of human endothelial cells derived from umbilical veins. J. Clin. Invest. **52**:2745-2756.

Jaffe, E.A. 1985. Physiologic functions of normal endothelial cells. Ann. NY Acad. Sci. **454**:279-291.

Jagielo, P.J., Quinn, T.J., Qureshi, N. and Schwartz, D.A. 1998. Grain dust-induced lung inflammation is reduced by *Rhodobacter sphaeroides* diphosphoryl lipid A. Am. J. Physiol. **274**:L26-L31.

Jahnke, A. and Johnson, J.P. 1994. Synergistic activation of intercellular adhesion molecule 1 (ICAM-1) by TNF-α and IFN-γ is mediated by p65/p50 and p65/c-Rel and interferon-responsive factor Stat1α (p91) that can be activated by both IFN-α and IFN-γ. FEBS Lett. **354**:220-226.

Jersmann, H.P.A., Hii. C.S.T., Hodge, G.L. and Ferrante, A. 2001. Synthesis and surface expression of CD14 by human endothelial cells. (*Infect. Immun..*, January 2001)

Jordana, M., Clancy, R, Dolovich, J, and Denburg, J. 1992. Effector role of the epithelial compartment in inflammation. Ann. N. Y. Acad. Sci. **664**:180-189.

Joseph, C.K., Wright, S.D., Bornmann, W.G., Randolph, J.T., Kumar, E.R., Bittman, R., Liu, J. and Kolesnick, R.N. 1994. Bacterial lipopolysaccharide has structural similarity to ceramide and stimulates ceramide-activated protein kinase in myeloid cells. J. Biol. Chem. **269**:17606-17610.

Juan, T.S.-C., Kelley, M.J., Johnson, D.A., Busse, L.A., Hailman, E., Wright, S.D. and Lichenstein, H.S. 1995. Soluble CD14 truncated at amino acid 152 binds lipopolysaccharide (LPS) and enables cellular responses to LPS. J. Biol. Chem. **270**:1382-1387. (a)

Juan, T.S.-C., Hailman, E., Kelley, M.J., Busse, L.A., Davy, E., Empig, C.J, Narhi, L.O., Wright, S.D. and Lichenstein, H.S. 1995. Identification of a lipopolysaccharide binding domain in CD14 between amino acids 57 and 64. J. Biol. Chem. **270**:5219-5224. (b)

Kanangat, S., Meduri, G.U., Tolley, E.A., Patterson, D.R., Meduri, C.U., Pak, C., Griffin, J.P., Bronze, M.S. and Schaberg, D.R. 1999. Effects of cytokines and endotoxin on the intracellular growth of bacteria. Infect. Immun. **67**:2834-2840.

Kane, J.P. and Havel, R.H. 1999. Polymorphism of the lipopolysaccharide receptor (CD14) and myocardial infarction. Circulation **99**:3210-3212.

Katz, S.S., Chen, K., Chen, S., Doerfler, M.E., Elsbach, P. and Weiss, J. 1996. Potent CD14-mediated signalling of human leukocytes by *Escherichia coli* can be mediated by interaction of whole bacteria and host cells without extensive prior release of endotoxin. Infect. Immun. **64**:3592-3600.

Kawai, T., Adachi, O., Ogawa, T., Takeda, K. and Akira, S. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity **11**:115-122.

Kawata, T., Bristol, J.R., Rose, J.R., Rossignol, D.P., Christ, W.J., Asano, O., Dubuc, G.R., Gavin, W.E., Hawkins, L.D., Kishi, Y., McGuinness, P.D., Mullarkey, M.A., Perez, M., Robidoux, A.L.C., Wang, Y., Kobayashi, S., Kimura, A., Katayama, K. and Yamatsu, I. 1995. Anti-endotoxin activity of a novel synthetic lipid A analog. Prog. Clin. Biol. Res. **392**:499-509.

Keicho, N., Elliott, W.M., Hogg, J.C. and Hayashi, S. 1997. Adenovirus E1A upregulates interleukin-8 expression induced by endotoxin in pulmonary epithelial cells. Am. J. Physiol. **272**:L1046-L1052.

Keller, III, G.E., Dey, R.D. and Burrell, R. 1991. Immunocytochemical determination of the role of alveolar macrophages in endotoxin processing in vitro and in vivo. Int. Arch. Allergy Appl. Immunol. **96**:149-155.

Khair, O.A., Devalia, J.L., Abdelaziz, M.M., Sapsford, R.J., Tarraf, H. and Davies, R.J. 1994. Effect of *Haemophilus influenzae* endotoxin on the synthesis of IL-6, IL-8, TNF-α and expression of ICAM-1 in cultured human bronchial epithelial cells. Eur. Resp. J. **7**:2109-2116.

Khair, O.A., Davies, R.J. and Devalia, J.L. 1996. Bacterial-induced release of inflammatory mediators by bronchial epithelial cells. Eur. Resp. J. **9**:1913-1922.

Khalil, N., O'Connor, R.N., Flanders, K.C., Shing, W. and Whitman, C.I. 1994. Regulation of type II alveolar epithelial cell proliferation by TGF-β during bleomycin-induced lung injury in rats. Am. J. Physiol. **267**:L498-L507.

Khirwadkar, K., Zilow, G., Oppermann, M., Kabelitz, D. und Rother, K. 1993. Interleukin-4 augments production of the third complement by the alveolar epithelial cell line A549. Int. Arch. Allergy Immunol. **100**:35-41.

Kingman, S. 1994. Resistance a European problem, too. Science **264**:363-365.

Kirschning, C.J., Wesche, H., Ayers, T.M. and Rothe, M. 1998. Human Toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. J. Exp. Med. **188**:2091-2097.

Klein, C.L., Köhler, H., Bittinger, F., Wagner, M., Hermanns, I., Grant, K., Lewis, J.C. and Kirkpatrick, C.J. 1994. Comparative studies on vascular endothelium in vitro. Pathobiol. **62**:199-208.

Kolesnik, R. and Golde, D.W. 1994. The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. Cell **77**:325-328.

Kollef, M.H. and Eisenberg, P.R. 1997. A rapid qualitative assay to detect circulating endotoxin can pedict the development of multiorgan dysfunction. Chest **112**:173-180.

Kopp, E.B. and Medzhitov, R. 1999. The Toll-receptor family and control of innate immunity. Curr. Opin. Immunol. **11**:13-18.

Kowanko, I.C. and Ferrante, A. 1987. Stimulation of neutrophil respiratory burst and lysosomal enzyme release by human interferon-gamma. Immunology **62**:149-151.

Krebs, E.G. 1994. The growth of research on protein phosphorylation. Trends Biochem. Sci. **19**:439.

Krüll, M., Dold, C., Hippenstiel, S., Rosseau, S., Lohmeyer, J. and Suttorp, N. 1996. *Escherichia coli* hemolysin and *Staphylococcus aureus* α-toxin potently induce neutrophil adhesion to cultured human endothelial cells. J. Immunol. **157**:4133-4140.

Kruger, M., Van De Winkel, J.G.J., De Wit, T.P.M., Coorevits, L. and Ceuppens, J.L. 1996. Granulocyte-macrophage colony stimulating factor down-regulates CD14 expression on monocytes. Immunology **89**:89–95.

Kuhns, D.B., Long Priel, D.A. and Gallin, J.I. 1997. Endotoxin and IL-1 hyporesponsiveness in a patient with recurrent bacterial infections. J. Immunol. **158**:3959-3964.

Kuijpers, T.W., Hakkert, B.C., Hart, M.H.L. and Roos, D. 1992. Neutrophil migration across monolayers of cytokine-prestimulated endothelial cells: a role for platelet-activating factor and IL-8. J. Cell Biol. **117**:565-572.

Kyriakis, J.M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E.A., Ahmad, M.F., Avruch, J. and Woodgett, J.R. 1994. The stress-activated protein kinase subfamily of c-jun kinases. Nature **369**:156-160.

Kyriakis, J.M. and Avruch, J. 1996. Sounding the alarm: protein kinase cascades activated by stress and inflammation. J. Biol. Chem. **271**:24313-24316.

Kyriakis, J.M. 1999. Signaling by the germinal center kinase family of protein kinases. J. Biol. Chem. **274**:5259-5262.

Landmann, R., Ludwig, C., Obrist, R. and Obrecht, J.P. 1991. Effect of cytokines and lipopolysaccharide on CD14 antigen expression in human monocytes and macrophages. J. Cell. Biochem. **47**:317-329.

Landmann, R., Zimmerli, W., Sansano, S., Link, S., Hahn, A., Glauser M.P. and Calandra, T. 1995. Increased circulating soluble CD14 is associated with high mortality in gram negative septic shock. J. Infect. Dis. **171**: 639-644.

Lauener, R.P., Goyert, S.M., Geha, R.S. and Vercelli, D. 1990. Interleukin 4 down-regulates the expression of CD14 in normal human monocytes. Eur. J. Immunol. **20**:2375-2381.

Lee, J.-D., Kato, K., Tobias, P.S., Kirkland, T.N. and Ulevitch, R.J. 1992. Transfection of CD14 into 70Z/3 cells dramatically enhances the sensitivity to complexes of lipopolysaccharide (LPS) and LPS binding protein. J. Exp. Med. **175**:1697-1705.

Lee, J.-D., Kravchenko, V., Kirkland, T.N., Han, J., Mackman, N., Moriarty, A., Leturcq, D., Tobias, P.S. and Ulevitch, R.J. 1993. Glycosyl-phosphatidylinositol-anchored or integral membrane forms of CD14 mediate identical cellular responses to endotoxin. Proc. Natl. Acad. Sci. USA **90**:9930-9934.

Lee, F.S., Hagler, J., Chen, Z.J. and Maniatis, T. 1997. Activation of the IκBα kinase complex by MEKK1, a kinase of the JNK pathway. Cell **88**:213-222.

Leeuwenberg, J.F.M., van Tits, L.J.H., Jeunhomme, T.M.A.A. and Buurman, W.A. 1995. Evidence for exclusive role in signalling of tumour necrosis factor p55 receptor and a potentiating function of p75 receptor on human endothelial cells. Cytokine **7**:457-462.

Leirisalo-Repo, M. 1994. The present knowledge of the inflammatory process and the inflammatory mediators. Pharmacol. Toxicol. **75** (Suppl. II):1-3.

Leturcq, D.J., Moriarty, A.M., Talbott, G., Winn, R.K., Martin, T.R. and Ulevitch, R.J. 1996. Antibodies against CD14 protect primates from endotoxin-induced shock. J. Clin. Invest. **98**:1533-1538.

Li, X.Y., Donaldson, K., Brown, D. and MacNee, W. 1995. The role of tumor necrosis factor in increased airspace epithelial permeability in acute lung inflammation. Am. J. Respir. Cell Mol. Biol. **13**:185-195.

Li, J.D., Feng, W., Gallup, M., Kim, J.H., Gum, J., Kim, Y. and Basbaum C. 1998. Activation of NF-kappaB via a Src-dependent Ras-MAPK-pp90rsk pathway is required for Pseudomonas aeruginosa-induced mucin overproduction in epithelial cells. Proc. Nat. Acad. Sci. USA **95**:5718-5723.

Lieber, M., Smith, B.T., Szakal, A., Nelson-Rees, W. and Todaro, G. 1976. A continuous tumour cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. Int. J. Cancer **17**:62-70.

Lienard, D., Ewalenko, P., Delmotte, J.-J., Renard, N. and Lejeune, F.J. 1992. High-dose recombinant tumor necrosis factor alpha in combination with interferon gamma and melphalan in isolation perfusion of the limbs for melanoma and sarcoma. J. Clin. Oncol. **10**:52-60.

Liu, Z.-G., Hsu, H., Goeddel, D.V. and Karin, M. 1996. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. Cell **87**:565-576.

Lloyd, A.R. and Oppenheim, J.J. 1992. Poly's lament: the neglected role of the polymorphnuclear neutrophil in the afferent limb of the immune response. Immunol. Today **13**:169-172.

Lopez, A. Shoji, S., Fujita, J., Robbins, R. and Rennard, S. 1988. Bronchepithelial cells can release hydrogen peroxide in response to inflammatory stimuli. Am. Rev. Respir. Dis. **137**:A81.

Lo, S.K., Van-Seventer, G.A., Levin, S.M. and Wright, S.D. 1989. Two leukocyte receptors (CD11a/CD18 and (CD11b/CD18) mediate transient adhesion to endothelium by binding to different ligands. J. Immunol. **143**:3325-3329.

Lozano, J., Berra, E., Municio, M.M., Diaz-Meco, M.T., Dominguez, I., Sanz, L. and Moscat, J. 1994. Protein kinase C ζ isoform is critical for κB-dependent promoter activation by sphingomyelinase. J. Biol. Chem. **269**:19200-19202.

Luchi, M. and Munford, R.S. 1993. Binding, internalization and deacylation of bacterial lipolysaccharide by human neutrophils. J. Immunol. **151**: 959-969.

Luo, Z.-J., Zhang, X.-F., Rapp, U. and Avruch, J. 1995. Identification of the 14.3.3 ζ domains important for self-association and Raf binding. J. Biol. Chem. **270**:23681-23687.

Mackay, F., Loetscher, H., Stueber, D., Gehr, G. and Lesslauer, W. 1993. Tumor necrosis factor α (TNF-α)-induced cell adhesion to human endothelial cells is under dominant control of one TNF receptor type, TNF-R55. J. Exp. Med. **177**:1277-1286.

MacKichan, M.L. and DeFranco, A.L. 1999. Role of ceramide in lipopolysaccharide (LPS)-induced signaling: LPS increases ceramide rather than acting as a structural homolog. J. Biol. Chem. **274**:1767-1775.

Malinin, N.L., Boldin, M.P., Kovalenko, A.V. and Wallach, D. 1997. MAP3K-related kinase involved in NF-κB induction by TNF, CD95 and IL-1. Nature **385**:540-544.

Manning, A.M., Bell, F.P., Rosenbloom, C.L., Chosay, J.G., Simmons, C.A., Northrup, J.L., Shebuski, R.J., Dunn, C.J. and Anderson, D.C. 1995. NF-kappa B is activated during acute inflammation in vivo in association with elevated endothelial adhesion molecule gene expression and leukocyte recruitment. J. Inflamm. **45**:283-296.

Marsh, C.B. and Wewers, M.D. 1996. The pathogenesis of sepsis: factors that modulate the response to gram-negative bacterial infection. Clin. Chest Med. **17**:183-197.

Martich, G.D., Danner, R.L., Ceska, M. and Suffredini, A.F. 1991. Detection of interleukin 8 and tumor necrosis factor in normal humans after intravenous endotoxin: the effect of antiinflammatory agents. J. Exp. Med. **173**:1021-1024.

Martin, T.R., Rubenfeld, G., Steinberg, K.P., Hudson, L.D., Raghu, G., Moriarty, A.M., Leturcq, D.J., Tobias, P. and Ulevitch, R.J. 1994. Endotoxin, endotoxin-binding protein, and soluble CD14 are present in bronchoalveolar lavage fluid of patients with adult respiratory distress syndrome. Chest **105**:S55-S56.

Martin, T.R., Rubenfeld, G.D., Ruzinski, J.T., Goodman, R.B., Steinberg, K.P., Leturcq, D.J., Moriarty, A.M., Raghu, G., Baughman, R.P. and Hudson, L.D. 1997. Relationship between soluble CD14, lipopolysaccharide binding protein, and the alveolar inflammatory inflammatory response in patients with acute respiratory distress syndrome. Am. J. Respir. Crit. Care Med. **155**:937-944.

Martin-Villa, J.M., Fere-Lopez, S., Lopez-Suarez, J.C., Corell, A., Perez-Blas, M. and Arnaiz-Villena, A. 1997. Cell surface phenotype and ultramicroscopic analysis of purified human enterocytes: a possible antigen-presenting cell in the intestine. Tissue Antigens **50**:586-592.

Masamune, A., Igarashi, Y. and Hakomori, S. 1996. Regulatory role of ceramide in interleukin (IL)-1β-induced E-selectin expression in human umbilical vein endothelial cells. J. Biol. Chem. **271**:9368-9375.

Matsuura, K., Ishida, T., Setoguchi, M., Higuchi, Y. and Yamamoto, S. 1993. Effect of antibodies against murine CD14 on LPS-induced TNF-α production in vitro and in vivo, and endotoxin shock. J. Leukocyte Biol. Suppl. 484 (Abstr.).

May, M.J., Wheeler-Jones, C.P., Houliston, R.A. and Pearson, J.D. 1998. Activation of p42mapk in human umbilical vein endothelial cells by interleukin-1 alpha and tumor necrosis factor-alpha. Am. J. Physiol.**274**:C789-C798.

Mayeux, P.R. 1997. Pathobiology of lipopolysaccharide. J. Toxicol. Envir. Health **51**:415-435.

McGinley, M.D., Narhi, L.O., Kelley, M.J., Davy, E., Robinson, J., Rohde, M.F., Wright, S.D. and Lichenstein, H.S. 1995. CD14: physical properties and identification of an exposed site that is protected by lipopolysaccharide. J. Biol. Chem. **270**:5213-5218.

Medzhitov, R., Preston-Hulburt, P. and Janeway, Jr, C.A. 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. Nature **388**:394-397.

Meijssen, M.A.C., Brandwein, S.L., Reinecker, H.C., Bhan, A.K. and Podolsky, D.K. 1998. Alteration of gene expression by intestinal epithelial cells precedes colitis in interleukin-2-deficient mice. Am. J. Physiol. **274**:G472-G479.

Meyer, C.F., Wang, X., Chang, C., Templeton, D. and Tan, T.-H. 1996. Interaction between c-Rel and the mitogen-activated protein kinase kinase kinase 1 signaling cascade in mediating κB enhancer activation. J. Biol. Chem. **271**:8971-8976.

Meyrick, B. and Brigham, K.L. 1986. Repeated *Escherichia coli* endotoxin-induced pulmonary inflammation causes chronic pulmonary hypertension in sheep. Lab. Invest. **55**:164-176.

Mickelson, J.K., Kukielka, G., Bravenec, J.S., Mainolfi, E., Rothlein, R., Hawkins, H.K., Kelly, J.H. and Smith, W. 1995. Differential expression and release of CD54 induced by cytokines. Hepatology **22**:866-875.

Milton, D.K., Godleski, J.J., Feldman, H.A. and Greaves, I.A. 1990. Toxicity of intratracheally instilled cotton dust, cellulose and endotoxin. Am. Rev. Respir. Dis. **142**:184-192.

Min, W. and Pober, J. 1997. TNF initiates E-selectin transcription in human endothelial cells through parallel TRAF-NF-κB and TRAF-rac/CDC42-JNK-c-JUN/ATF2 pathways. J. Immunol. **159**:3508-3518.

Modlin, R.L., Brightbill, H.D. and Godowski, P.J. 1999. The toll of innate immunity on microbial pathogens. N. Engl. J. Med. **340**:1834-1835.

Modur, V., Zimmerman, G.A., Prescott, S.M. and McIntyre, T.M. 1996. Endothelial cell inflammatory responses to tumor necrosis factor- α. J. Biol. Chem. **271**:13094-13102.

Mohler, K.M., Torrance, D.S., Smith, C.A., Goodwin, R.G., Stremler, K.E., Fung, V.P., Madani, H. and Widmer, M.B. 1993. Soluble tumor necrosis factor (TNF) receptors are effective therapeutic agents in lethal endotoxemia and function simultaneously as both TNF carriers and TNF antagonists. J. Immunol. **151**:1548-1561.

Montgomery, K.F., Osborn, L., Hession, C., Tizard, R., Goff, D., Vassallo, C., Tarr, P.I., Bomsztyk, K., Lobb, R., Harlan, J.M. and Pohlman, T.H. 1991. Activation of endothelial-leukocyte adhesion molecule 1 (ELAM-1) gene transcription. Proc. Natl. Acad. Sci. USA **88**:6523-6527.

Moodie, S.A., Willumsen, B.M., Weber, M.J. and Wolfman, A. 1993. Complexes of Ras·GTP with Raf-1 and mitogen-activated protein kinase kinase. Science **260**:16581661.

Moreira, A.L., Wang, J., Sarno, E.N. and Kaplan, G. 1997. Thalidomide protects mice against LPS-induced shock. Braz. J. Med. Biol. Res. **30**:1199-1207.

Mulligan, M.S., Varani, J., Dame, M.K., Lane, C.L., Smith, C.W., Anderson, D.C. and Ward, P.A. 1991. Role of endothelial-leukocyte adhesion molecule 1 (ELAM-1) in neutrophil-mediated lung injury in rats. J. Clin. Invest. **88**:1396-1406.

Murray, H.W. 1988. Interferon-γ, the activated macrophage, and host defense against microbial challenge. Ann. Int. Med. **108**:595-608.

Nakamurra, H., Yoshimura, K., Jaffe, H.A. and Crystal, R.G. 1991. Interleukin-8 gene expression in human bronchial epithelial cells. J. Biol. Chem. **266**:19611-19617.

Nardone, L.L. and Andrews, S.B. 1979. Cell line A549 as a model of the type II pneumocyte. Biochim. Biophys. Acta **573**:276-295.

Nathan, C.F. 1987. Neutrophil activation on biological surfaces: massive secretion of hydrogen peroxide in response to products of macrophages and lymphocytes. J. Clin. Invest. **80**:1550–1560.

Natoli, G., Costanzo, A., Ianni, A., Templeton, D.J., Woodgett, J.R., Balsano, C. and Levrero, M. 1997. Activation of SAPK/JNK by TNF receptor 1 through a noncytotoxic TRAF2-dependent pathway. Science **275**:200-203.

Nelson, D., Kuppermann, N., Fleisher, G.R., Hammer, B.K., Thompson, C.M., Garcia, C.T., Novitsky, T.J., Parsonett, J., Onderdonk, A., Siber, G.R. and Saladino, R.A. 1995. Recombinant endotoxin neutralizing protein improves survival from *Escherichia coli* sepsis in rats. Crit. Care Med. **23**:92-98.

Nelson, S., Belknap, S.M., Carlson, R.W., Dale, D., DeBoisblanc, B., Farkas, S., Fotheringham, N., Ho, H., Marrie, T., Movahhed, H., Root, R. and Wilson, J. 1998. A randomized controlled trial of Filgrastim as an adjunct to antibiotics for treatment of hospitalized patients with community-acquired pneumonia. J. Infect. Dis. **178**:1075-1080.

Newton, R., Adcock, I.M. and Barnes, P.J. 1995. Superinduction of NF-κB by actinomycin D and cycloheximide in epithelial cells. Biochem. Biophys. Res. Commun. **218**:518-523.

Nick, J.A., Avdi, N.J., Gerwins, P., Johnson, G.L. and Worthen, G.S. 1996. Activation of a p38 mitogen-activated protein kinase in human neutrophils by lipopolysaccharide. J. Immunol. **156**:4867-4875.

Nick, J.A., Avdi, N.J., Young, S.K., Lehman, L.A., McDonald, P.P., Frasch, S.C., Billstrom, M.A., Henson, P.M., Johnson, G.L. and Worthen, G.S. 1999. Selective activation and functional significance of p38α mitogen-activated protein kinase in lipopolysaccharide-stimulated neutrophils. J. Clin. Invest. **103**:851-858.

Nishikawa, K., Arai, H. and Inoue, K. 1990. Scavenger receptor-mediated uptake and metabolism of lipid vesicles containing acidic phospholipids by mouse peritoneal macrophages. J. Biol. Chem. **265**:5226-5231.

Noel, R.F., Jr., Sato, T.T., Mendez, C., Johnson, M.C. and Pohlman, T.H. 1995. Activation of human endothelial cells by viable or heat-killed gram-negative bacteria requires soluble CD14. Infect. Immun. **63**:4046-4053.

Novogrodsky, A., Vanichkin, A., Patya, M., Gazit, A., Osherov, N. and Levitzki, A. 1994. Prevention of lipopolysaccharide-induced lethal toxicity by tyrosine kinase inhibitors. Science **264**:1319-1322.

Nuorti, J.P., Butler, J.C., Crutcher, J.M., Guevara, R., Welch, D., Holder, P. and Elliott, J.A. 1998. An outbreak of multidrug-resistant pneumococcal pneumonia and bacteremia among unvaccinated nursing home residents. N. Engl. J. Med. **338**:1861-1868.

O'Connell, M.A., Bennett, B.L., Mercurio, F., Manning, A.M. and Mackman, N. 1998. Role of IKK1 and IKK2 in lipopolysaccharide signaling in human monocytic cells. J. Biol. Chem. **273**:30410-30414.

Ohlson, K., Björk, P., Bergenfeldt, M., Hageman, R. and Thompson, R.C. 1990. Interleukin-1 receptor antagonist reduces mortality from endotoxin shock. Nature **348**:550-552.

O'Neill, L.A.J. and Greene, C. 1998. Signal transduction pathways activated by the IL-1 receptor family: ancient signaling machinery in mammals, insects and plants. J. Leukoc. Biol. **63**:650-657.

Onozuka, K., Kirikae, T., Kirikae, F., Suda, Y., Kusumoto, S., Yamamoto, S., Shimamura, T. and Nakano, M. 1997. Participation of CD14 in the phagocytosis of smooth-type *Salmonella typhimurium* by the macrophage-like cell line, J774.1. Microbiol. Immunol. **41**:765-772.

Opal, S.M., Cross, A.S., Jhung, J.W., Young, L.D., Palardy, J.E., Parejo, N.A. and Donsky, C. 1996. Potential hazards of combination immunotherapy in the treatment of experimental septic shock. J. Infect. Dis. **173**:1415-1421.

Osborn, L., Kunkel, S. and Nabel, G.J. 1989. Tumor necrosis factor-α and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor κB. Proc. Natl. Acad. Sci. USA **86**:2336-2340. (a)

Osborn, L., Hession, C., Tizard, R., Vassallo, C., Luhowskyj, S., Chi-Rosso, G. and Lobb, R. 1989. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell **59**:1203-1211. (b)

Palmberg, L., Larsson, B.M., Malmberg, P. and Larsson, K. 1998. Induction of IL-8 production in human alveolar macrophages and human bronchial epithelial cells in vitro by swine dust. Thorax **53**:260-264.

Pan, L.-F., Kreisle, R.A. and Shi, Y.-D. 1998. Detection of Fcγ receptors on human endothelial cells stimulated with cytokines tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) Clin. Exp. Immunol. **112**:533-53.

Peterson, P.K., Gekker, G., Hu, S., Sheng, W.S., Anderson, W.R., Ulevitch, R.J., Tobias, P.S., Gustafson, K.V., Molitor, T.W. and Chao, C.C. 1995. CD14 receptor-mediated uptake of nonopsonized *Mycobacterium tuberculosis* by human microglia. Infect. Immun. **63**:1598-1602.

Pober, J.S. and Cotran, R.S. 1990. Cytokines and endothelial cell biology. Physiol. Rev. **70**:427-451.

Pober, J.S. 1998. Activation and injury of endothelial cells by cytokines. Pathol. Biol. Paris **46**:159-163.

Pollack, M. and Ohl, C.A. 1996. Endotoxin-based molecular stratefies for the prevention and treatment of gram-negative sepsis and septic shock. Curr. Top. Microbiol. Immunol. **216**:275-297.

Poltorak, A., He, X., Smirnova, I., Liu, M.-Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Riccardi-Castagnoli, P., Layton, B. and Beutler, B. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene. Science **282**:2085-2088.

Pombo, C.M., Kehrl, J.H., Sánchez, I., Katz, P., Avruch, J., Zon, L.I., Woodgett, J.R., Force, T. and Kyriakis, J.M. 1995. Activation of the SAPK pathway by the human STE20 homologue germinal kinase. Nature **377**:750-754.

Pottratz, S.T., Paulsrud, J., Smith, J.S. and Martin II, W.J. 1991. Pneumocystis carinii attachment to cultured lung cells by pneumocystis gp120, a fibronectin binding protein. J. Clin. Invest. **88**:403-407.

Pugin, J., Schürer-Maly, C.C., Leturcq, D., Moriarty, A., Ulevitch, R.J. and Tobias, P.S. 1993. Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. Proc. Natl. Acad. Sci. USA **90**:2744-2748.

Pugin, J., Ulevitch, R.J. and Tobias, P.S. 1993. A critical role for monocytes and CD14 in endotoxin-induced endothelial cell activation. J. Exp. Med. **178**:2193-2200.

Quezado, Z.M.N., Natanson, C., Banks, S.M., Alling, D.W., Koev, C.A., Danner, R.L., Elin, R.J., Hosseini, J.M., Parker, T.S., Levine, D.M., Rubin, A.L. and Hoffman, W.D. 1995. Therapeutic trial of reconstituted human high-density lipoprotein in a canine model of gram-negative shock. J. Pharmacol. Exp. Ther. **272**:604-611.

Qin, L., Quinlan, W.M., Doyle, N.A., Graham, L., Sligh, J.E., Takei, F., Beaudet, A.L. and Doerschuk, C.M. 1996. The roles of CD11/CD18 and ICAM-1 in acute *Pseudomonas aeruginosa*-induced pneumonia in mice. J. Immunol. **157**:5016-5021.

Ray, K.P. and Kennard, N. 1993. Interleukin-1 induces a nuclear form of transcription factor NF-κB in human lung epithelial cells. Agents Actions **38**:C61-C63.

Read, M.A., Whitley, M.Z., Williams, A.J. and Collins, T. 1994. NF-κB and IκBα: An inducible regulatory system in endothelial activation. J. Exp. Med. **179**:503-512.

Read, M.A., Whitley, M.Z., Gupta, S., Pierce, J., Best, J., Davis, R.J. and Collins, T. 1997. Tumor necrosis factor α-induced E-selectin expression is activated by the nuclear factor-κB and c-JUN N-terminal kinase/p38 mitogen-activated protein kinase pathways. J. Biol. Chem. **272**:2753-2761.

Régnier, C.H., Song, H.Y., Gao, X., Goeddel, D.V., Cao, Z. and Rothe, M. 1997. Identification and characterization of an IκB kinase. **90**:373-383.

Rehm, S.R., Gross, G.N. and Pierce, A.K. 1980. Early bacterial clearance from murine lungs. J. Clin. Invest. **66**:194-199.

Reimann, T., Büscher, D, Hipskind, R.A., Krautwald, S., Lohmann-Matthes, M.-L. and Baccarini, M. 1994. Lipopolysaccharide induces activation of the Raf-1/MAP kinase pathway. J. Immunol. **153**:5740-5749.

Reimold, A.M., Grusby, M.J., Kosaras, B., Fries, J.W.U., Mori, R., Maniwa, S., Clauss, I.M., Collins, T., Sidman, R.L., Glimcher, M.J. and Glimcher, L.H. 1996. Chondrodysplasia and neurological abnormalities in ATF-2 deficient mice. Nature **379**:262-265.

Richter, J., Andersson, T. and Olsson, I. 1989. Effect of tumor necrosis factor and granulocyte/macrophage colony-stimulating factor on neutrophil degranulation. J. Immnuol. **142**:3199-3205.

Rietschel, E.T. and Brade, H. 1992. Bacterial endotoxins. Sci. Am. **267**:26-33.

Rietschel, E.Th., Schletter, J., Weidemann, B., El-Samalouti, V., Mattern, T., Zahringer, U., Seydel, U., Brade, H., Flad, H.-D., Kusumoto, S., Gupta, D, Dziarski, R. and Ulmer, A.J. 1998. Lipopolysaccharide and peptidoglycan: CD14-dependent bacterial inducers of inflammation. Microb. Drug. Resist. **4**:37-44.

Ritchie, A.J., Johnson, D.R., Ewenstein, B.M. and Pober, J.S. 1991. Tumor necrosis factor induction of endothelial cell surface antigens is independent of protein kinase C activation or inactivation. Studies with phorbol myristate acetate and stauroporine. J. Immunol. **146**:3056-3062.

Robinson, P.J. 1991. Phosphatidyl inositol membrane anchors and T-cell activation. Immunol. Today. **12**:35-41.

Rock, F.L., Hardiman, G., Timans, J.C., Kastelein, R.A. and Bazan, J.F. 1998. A family of human receptors structurally related to *Drosophila* Toll. Proc. Natl. Acad. Sci. USA **95**:588-593.

Rodeberg, D.A., Morris, R.E. and Babcock, G.F. 1997. Azurophilic granules of human neutrophils contain CD14. Infect. Immun. **65**:4747-4753.

Roth, R.I., Kaca, W. and Levin, J. 1994. Hemoglobin: a newly recognized binding protein for bacterial endotoxins (LPS). Prog. Clin. Biol. Res. **388**:161-172.

Rothman, B.L., Merrow, M., Despins, A., Kennedy, T. and Kreutzer, D.L. 1989. Effect of lipopolysaccharide on C3 and C5 production by human lung cells. J. Biol. Chem. **143**:196-202.

Rubens, C.E., Smith, S., hulse, M., Chi, E. and van Belle, G. 1992. Respiratory epithelial cell invason by group B streptococci. Infect. Immun. **60**:5157-5163.

Rustici, A., Velucchi, M., Faggioni, R., Sironi, M., Ghezzi, P., Quataert, S., Green, B. and Porro, M. 1993. Molecular mapping and detoxification of the lipid A binding site by synthetic peptides. Science **259**:361-365.

Rutenburg, S.H., Schweinburg, F.B. and Fine, J. 1960. In vitro detoxification of bacterial endotoxin by macrophages. J. Exp. Med. **112**:801-807.

Rylander, R. 1995. Endotoxins in the environment. Prog. Clin. Biol. Res. **392**:79-90.

Saadi, A.T., Blackwell, C.C., Essery, S.D., Raza, M.W., El Ahmer, O.R., MacKenzie, D.A.C., James, V.S., Weir, D.M., Ogilvie, M.M., Elton, R.A., Busuttil, A. and Keeling, J.W. 1996. Developmental and environmental factors that enhance binding of *Bordetella pertussis* to human epithelial cells in relation to sudden infant death syndrome (SIDS) FEMS Immunol. Med. Microbiol. **16**:51-59.

Sallenave, J.-M., Silva, A., Marsden, M.E. and Ryle, A.P. 1993. Secretion of mucus proteinase inhibitor and elafin by Clara cell and type II pneumocyte cell lines. Am. J. Respir. Cell Mol. Biol. **8**:126-133.

Sánchez, I., Hughes, R.T., Mayer, B.J., Yee, K., Woodgett, J.R., Avruch, J., Kyriakis, J.M. and Zon, L.I. 1994. Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. Nature 372:794-798.

Sandström, T., Bjermer, L. and Rylander, R. 1992. Lipopolysaccharide (LPS) inhalation in healthy subjects increases neutrophils, lymphocytes and fibronectin levels in bronchoalveolar lavage fluid. Eur. Respir. J. 5:992-996.

Sariban, E., Sitaras, N.M., Antoniades, H.N., Kufe, D.W. and Pantazis, P. 1988. Expression of platelet-derived growth factor (PDGF)-related transcripts and synthesis of biologically active PDGF-like proteins by human malignant epithelial cell lines. J. Clin. Invest. 82:1157-1164.

Savill, J., Dransfield, I., Hogg, n. and Haslett, C. 1990. Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis. Nature 343:170-173.

Savill, J., Hogg, N., Ren, Y. and Haslett, C. 1992. Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis. J. Clin. Invest. 90:1513-1522.

Schimke, J., Mathison, J., Morgiewicz, J. and Ulevitch, R.J. 1998. Anti-CD14 mAb treatment provides therapeutic benefit after *in vivo* exposure to endotoxin. Proc. Natl. Acad. Sci. USA 95:13875-13880.

Schipper, H., Krohne, G.F. and Gross, R. 1994. Epithelial cell invasion and survival of *Bordetella bronchiseptica*. Infect. Immun. 62:3008-3011.

Schletter, J., Brade, H., Brade, L., Krueger, C., Loppnow, H., Kusumoto, S., Rietschel, E., Flad, H.-D. and Ulmer, A.J. 1995. Binding of Lipopolysaccharide (LPS) to an 80-kilodalton membrane protein of human cells is mediated by soluble CD14 and LPS-binding protein. Infect. Immun. 63:2576-2580.

Schmid, I., Uittenbogaart, C.H., Keld, B. and Giorgi, J.V. 1994. A rapid method for measuring apoptosis and dual-color immunofluorescence by single laser flow cytometry. J. Immunol. Meth. 170:145-157.

Schmitz, M.L., dos Santos Silva, M.A., and Baeuerle, P.A. 1995. Transactivation Domain (TA$_2$) of p65 NF-κB. J. Biol. Chem. 270:15576-15584.

Schulze-Osthoff, K, Ferrari, D., Riehemann, K. and Wesselborg, S. 1997. Regulation of NF-κB activation by MAP kinase cascades. Immunobiol. **198**:35-49.

Schumann, R.R., Pfeil, D., Lamping, N., Kirschning, C., Scherzinger, G., Schlag, P., Karawajew, L. and Herrmann, F. 1996. Lipopolysaccharide induces the rapid tyrosine phosphorylation of the mitogen-activated protein kinases erk-1 and p38 in cultured human vascular endothelial cells requiring the presence of soluble CD14. Blood **87**:2805-2814.

Schussheim, A.E. and Fuster, V. 1999. Antibiotics for myocardial infarction ? Drugs **57**:283-291.

Schütt, C., Ringel, B., Nausch, M., Bazil, V., Horejsi., V., Neels, P., Walzel, H., Jonas, L., Siegl, E., Friemel, H. and Plantikow, A. 1988. Human monocyte activation induced by an anti-CD14 monoclonal antibody. Immunol. Lett. **19**:321-328.

Schütt, C., Schilling, T., Grunwald, U., Schoenfeld, W. and Krüger, C. 1992. Endotoxin-neutralizing capacity of soluble CD14. Res. Immunol. **143**:71-78.

Sengeløv, H., Follin, P., Kjeldsen, L., Lollike, K., Dahlgren, C. and Borregaard, N. 1995. Mobilization of granules and secretory vesicles during in vivo exudation of human neutrophils. J. Immunol. **154**:4157-4165.

Setoguchi, M., Nasu, N., Yoshida, S., Higuchi, Y., Akizuki, S. and Yamamoto, S. 1989. Mouse and human CD14 (myeloid cell-specific leucine-rich glycoprotein) primary structure deduced from cDNA clones. Biochim. Biophys. Acta **1008**:213-222.

Shalaby, M.R., Aggarwal, B.B., Rinderknecht, E., Svedersky, L.P., Finkle, B.S. and Palladino, M.A. Jr. 1985. Activation of human polymorphonuclear neutrophil functions by interferon-γ and tumour necrosis factors. J. Immunol. **135**:2069 -2073.

Shall, T.J. and Bacon, K.B. 1994. Chemokines, leukocyte trafficking, and inflammation. Curr. Opin. Immunol. **6**:865-873.

Sharma, S.A., Olchowy, T.W.J., Yang, Z. and Breider, M.A. 1992. Tumor necrosis factor α and interleukin 1 α enhance lipopolysaccharide-mediated bovine endothelial cell injury. J. Leukoc. Biol. **51**:579-585.

Shepherd, V.L. 1991. Cytokine receptors of the lung. Am. J. Respir. Cell Mol. Biol. **5**:403-410.

Shi, C.-S. and Kehrl, J.H. 1997. Activation of stress-activated protein kinase/c-jun N-terminal kinase, but not NF-κB, by the tumor necrosis factor (TNF) receptor 1 through a TNF receptor-associated factor 2- and germinal center kinase related-dependent pathway. J. Biol. Chem. **272**:32102-32107.

Sibille, Y. and Reynolds, H.Y. 1990. Macrophages and pulmonary neutrophils in lung defense and injury. Am. Rev. Respir. Dis. **141**:471-501.

Siegel, S.A., Shealy, D.J., Nakada, M.T., Le, J., Woulfe, D.S., Probert, L., Kollias, G., Ghrayeb, J., Vilcek, J. and Daddona, P.E. 1995. The mouse/human chimeric monoclonal antibody cA2 neutralizes TNF in vitro and protects transgenic mice from cachexia and TNF lethality in vivo. Cytokine **7**:15-25.

Simon, R.H. and Paine III, R. 1995. Participation of pulmonary alveolar epithelial cells in lung inflammation. J. Lab. Clin. Med. **126**:108-118.

Simons, K. and Ikonen, E. 1997. Functional rafts in cell membranes. Nature **387**:569-572.

Slowik, M.R., De Luca, L.G., Min, W. and Pober, J.S. 1996. Ceramide is not a signal for tumor necrosis factor-induced gene expression but does cause programmed cell death in human vascular endothelial cells. Circ. Res. **79**:736-747.

Smart, S.J. and Casale, T.B. 1994. Pulmonary epithelial cells facilitate TNF-α-induced neutrophil chemotaxis. J. Immunol. **152**:4087-4094.

Smith, J.A. 1994. Neutrophils, host defense, and inflammation: a double-edged sword. J. Leukoc. Biol. **56**:672-686.

Smith, B.T. 1977. Cell line A 549: a model system for the study of alveolar type II cell function. Am. Rev. Resp. Dis. **115**:285-293.

Song, H.Y., Régnier, C.H., Kirschning, C.J., Goeddel, D.V. and Rothe, M. 1997. Tumor necrosis factor (TNF)- mediated kinase cascades: bifurcation of nuclear factor-κB and c-jun N-terminal kinase (JNK/SAPK) pathways at TNF receptor-associated factor 2. Proc. Natl. Acad. Sci. USA **94**:9792-9796.

Springer, T.A. 1990. Adhesion receptors of the immune system. Nature **346**:425-434.

Standiford, T.J., Kunkel, S.L., Basha, M.A., Chensue, S.W., Lynch III, J.P., Toews, G.B., Westwick, J. and Strieter, R.M. 1990. Interleukin-8 gene expression by a pulmonary epithelial cell line. A model for cytokine networks in the lung. J. Clin. Invest. **86**:1945-1953.

Standiford, T.J., Kunkel, S.L., Phan, S.H., Rollins, B.J. and Strieter, R.M. 1991. Alveolar macrophage derived cytokines induce monocyte chemoattractant protein-1 expression from human pulmonary type II-like epithelial cells. J. Biol. Chem. **266**:9912-9918.

Staubach, K.H., Schroder, J., Stuber, F., Gehrke, K., Traumann, E. and Zabel, P. 1998. Effect of pentoxifylline in severe sepsis: results of a randomized, double-blind, placebo-controlled study. Arch. Surg. **133**:94-100.

Steadman, R., Petersen, M.M. and Williams, J.D. 1996. Human neutrophil secondary granule exocytosis in independent of protein kinase activation and is modified by calmodulin activity. Int. J. Biochem. Cell Biol. **28**:777-786.

Stefanova, I., Horejsi, V., Ansotegui, I.J., Knapp, W. and Stockinger, H. 1991. GPI-anchored cell surface molecules complexed to protein tyrosine kinases. Science **254**:1016-1019.

Stefanova, I., Corcoran, M.L., Horak, E.M., Wahl, L.M., Bolen, J.B. and Horak, I.D. 1993. Lipopolysaccharide induces activation of CD14-associated protein tyrosine kinase $p53/56^{lyn}$. J. Biol. Chem. **268**:20725-20728.

Stellato, C., Beck, L.A., Gorgone, G.A., Proud, D., Schall, T.J., Ono, S.J., Lichtenstein, L.M. and Schleimer, R.P. 1995. Expression of the chemokine RANTES by a human bronchial epithelial cell line. J. Immunol. **155**:410-418.

Stelter, F., Bernheiden, M., Menzel, R., Jack, R.S., Witt, S., Fan, X., Pfister, M. and Schütt, C. 1997. Mutation of amino acids 39-44 of human CD14 abrogates binding to lipopolysaccharide and Escherichia coli. Eur. J. Biochem. **243**: 100-109.

Stern, A., Engelhardt, R., Foster, C., Golenbock, D., Hildebrandt, J., Landmann, R., Mayer, P., and Stütz, P. 1995. SDZ MRL 953, a lipid A analog as selective cytokine inducer. Prog. Clin. Biol. Res. **392**:549-565.

Stone, R. 1994. Search for sepsis drugs goes on despite past failures. Science **264**:365-367.

Strieter, R.M., Kasahara, K., Allen, R.M., Standiford, T.J., Rolfe, M.W., Becker, F.S., Chensue, S.W. and Kunkel, S.L. 1992. Cytokine-induced neutrophil-derived interleukin-8. Am. J. Pathol. **141**:397-407.

Stríz, I., Mio, T., Adachi, Y., Bazil, V. and Rennard, S. 1998. The CD14 molecule participates in regulation of IL-8 and IL-6 release by bronchial epithelial cells. Immunol. Lett. **62**:177-181.

Suffredini, A.F., Fromm, R.E., Parker, M.M., Brenner, M., Kovacs, J.A., Wesley, R.A. and Parrillo, J.E. 1989. The cardiovascular response of normal humans to the administration of endotoxin. N. Engl. J. Med. **321**:280-287.

Sweet, M.J. and Hume, D.A. 1996. Endotoxin signal transduction in macrophages. J. Leukoc. Biol. **60**:8-26.

Takaoka, A., Tanaka, N., Mitani, Y., Miyazaki, T., Fujii, H., Sato, M., Kovarik, P., Decker, T., Schlessinger, J. and Taniguchi, T. 1999. Protein tyrosine kinase Pyk2 mediates the Jak-dependent activation of MAPK and Stat1 in IFN-γ, but not IFN-α, signaling. EMBO J. **18**:2480-2488.

Takeda, J., Miyata, T., Kawagoe, K., Iida, Y., Endo, Y., Fujita, T., Takahashi, M., Kitani,T. and Kinoshita, T. 1993. Deficiency of the GPI anchor caused by a somatic mutation of the *PIG-A* gene in paroxysmal nocturnal hemoglobinuria. Cell **73**:703-711.

Takeshita,, S., Nakatani, K., Takata, Y., Kawase, H., Sekine, I. and Yoshioka, S. 1998. Interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) enhance lipopolysaccharide binding to neutrohils via CD14. Inflamm. Res. **47**:101-103.

Talbot, U.M., Paton, A.W. and Paton, J.C. 1996. Uptake of *Streptococcus pneumoniae* by respiratory epithelial cells. Infect. Immun. **64**:3772-3777.

Tamura, D.Y., Moore, E.E., Johnson, J.L., Zallen, G., Aiboshi, J. and Silliman, C.C. 1998. P38 mitogen-activated protein kinase inhibition attenuates intercellular adhesion molecule-1 Up-regulation on human pulmonary microvascular endothelial cells. Surgery **124**:403-408.

Tasaka, S., Ishizaka, A., Urano, T., Sayama, K., Sakamaki, F., Nakamura, H., Terashima, T., Waki, Y., Soejima, K., Nakamura, M., Matsubara, H., Fujishima, T., Kanazawa, M. and Larrick, J.W. 1996. A derivative of cationic antimicrobial protein attenuates lung injury by suppressing cell adhesion. Am. J. Respir. Cell Mol. Biol. **15**:738-744.

Taveira da Silva, A.M., Kaulbach, H.C., Chuidian, F.S., Lambert, D.R., Suffredini, A.F. and Danner, R.L. 1993. Brief report: shock and multiple-organ dysfunction after self-administration of salmonella endotoxin. N. Engl. J. Med. **328**:1457-1460.

Teng, N.N.H., Kaplan, H.S., Hebert, J.M., Moore, C., Douglas, H., Wunderlich, A. and Braude, A.I. 1985. Protection against gram-negative bacteremia and endotoxemia with human monoclonal IgM antibodies. Proc. Natl. Acad. Sci. USA **82**:1790-1794.

Thieblemont, N. and Wright, S.D. 1999. Transport of bacterial lipopolysaccharide to the Golgi apparatus. J. Exp. Med. **190**:523-534.

Thomas, J.A., Alen, J.L., Tsen, M., Dubnicoff, T., Danao, J., Liao, X.C., Cao, Z. and Wasserman, S.A. 1999. Impaired cytokine signaling in mice lacking the IL-1 receptor-associated kinase. J. Immunol. **163**:978-984.

Tobias, P.S., Mathison, J.C. and Ulevitch, R.J. 1988. A family of lipopolysaccharide binding proteins involved in responses to gram-negative sepsis. J. Biol. Chem. **264**:13479-13481.

Tobias, P.S., Mathison, J., Mintz, D., Lee, J.-D., Kravchenko, V., Kato, K., Pugin, J. and Ulevitch, R.J. 1992. Participation of lipopolysaccharide-binding protein in lipopolysaccharide-dependent macrophage activation. Am. J. Respir. Cell Mol. Biol. **7**:239-245.

Tobias, P.S. and Ulevitch, R.J. 1994. Lipopolysaccharide-binding protein and CD14 in the lipolpolysaccharide-dependent activation of cells. Chest **105**:48s-50s.

Toews, G.B. 1986. Determinants of bacterial clearance from the lower respiratory tract. Sem. Respir. Infect. **1**:68-78.

Tohme, Z.N., Amar, S. and van Dyke, T.E. 1999. Moesin functions as a lipopolysaccharide receptor on human monocytes. Infect. Immun. **67**:3215-3220.

Tosi, M.F., Stark, J.M., Smith, C.W., Hamedani, A., Gruenert, D.C. and Infeld, M.D. 1992. Induction of ICAM-1 expression on human airway epithelial cells by inflammatory cytokines: effects on neutrophil-epithelial cell adhesion. Am. J. Respir. Cell Mol. Biol. **7**:214-221.

Traenckner, E.B.-M., Pahl, H.L., Henkel, T., Schmidt, K.N., Wilk, S. and Baeuerle, P.A. 1995. Phosphorylation of human IκB-α on serines 32 and 36 controls IκB-α proteolysis and NF-κB activation in response to diverse stimuli. EMBO J. **14**:2876-2883.

Troelstra, A., Antal-Szalmas, P., De Graaf-Miltenburg, L.A.M., Trubetskoy, V.S., Koshkina, N.V., Omel'yanenko, V.G., L'vov, V. L., Dmitriev, B.A., Petrov, A.B. and Torchilin, V.P. 1990. FITC-labeled lipopolysaccharide: use as a probe for liposomal membrane incorporation studies. FEBS-Lett. **269**: 79-80.

Tsujimoto, M., Yip, Y.K. and Vilcek, J. 1986. Interferon-γ enhances expression of cellular receptors for tumor necrosis factor. J. Immunol. **136**:2441-2444.

Turner, R.B., Wecker, M.T., Pohl, G., Witek, T.J., McNally, E., St. George, R., Winther, B. and Hayden, F.G. 1999. Efficacy of tremacamra, a soluble intercellular adhesion molecule 1, for experimental rhinovirus infection. JAMA **281**:1797-1804.

Ulevitch, R.J., Johnston, A.R. and Weinstein, D.B. 1979. New function for high density lipoproteins. J. Clin. Invest. **64**:1516-1524.

Ulevitch, R.J. and Tobias, P.S. 1994. Recognition of endotoxin by cells leading to transmembrane signaling. Curr. Opin. Immunol. **6**:125–130.

Ulevitch, R.J. and Tobias, P.S. 1995. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. Annu. Rev. Immunol. **13**:437–457.

Ulevitch, R.J. and Tobias, P.S. 1999. Recognition of gram-negative bacteria and endotoxin by the innate immune system. Curr. Opin. Immunol. **11**:19-22.

Unkelbach, K., Gardemann, A., Kostrzewa, M., Philipp, M., Tillmann, H. and Haberbosch, W. 1999. A new promoter polymorphism in the gene of lipopolysaccharide receptor CD14 is associated with expired myocardial infarction in patients with low atherosclerotic risk profile. Arterioscler. Thromb. Biol. **19**:932-938.

Vacheron, F., Mandine, E., Lenaour, R., Smets, P., Zalisz, R. and Guenounou, M. 1992. Inhibition of production of tumor necrosis factor by monoclonal antibodies to lipopolysaccharides. J. Infect. Dis. **165**:873-878.

Van der Bruggen, T., Nijenhuis, S., Van Raaij, E., Verhoef, J. and Van Asbeck, B.S. 1999. Lipopolysaccharide-induced tumor necrosis factor alpha production by human monocytes involves the Raf-1/MEK-1-MEK2/ERK1-ERK2 pathway. Infect. Immun. **67**:3824-3829.

Van Deventer, S.J.H., Büller, H.R., ten Cate, J.W., Aarden, L.A., Hack, C.E. and Sturk, A. 1990. Experimental endotoxemia in humans: analysis of cytokine release and coagulation, fibrinolytic, and complement pathways. Blood **76**:2520-2526.

Van Voorhis, W.C., Steinman, R.M., Hair, L.S., Luban, J., Witmer, M.D., Koide, S. and Cohn, Z.A. 1983. Specific antimononuclear phagocyte monoclonal antibodies. Application to the purification of dendritic cells and the tissue location of macrophages. J. Exp. Med. **158**:126-145.

Vane, J.R., Änggård, E.E. and Botting, R.M. 1990. Regulatory functions of the vascular endothelium. N. Eng. J. Med. **323**:27-34.

Vasselon, T., Pironkova, R. and Detmers, P. 1997. Sensitive responses of leukocytes to lipopolysaccharide require a protein distinct from CD14 at the cell surface. J. Immunol. **159**:4498-4505.

Vasselon, T., Hailman, E., Thieringer, R. and Detmers, P.A. 1999. Internalization of monomeric lipopolysaccharide occurs after transfer out of cell surface CD14. J. Exp. Med. **190**:509-521.

Vincent, J.-L. 1997. New therapies in sepsis. Chest **112**:330S-338S.

Von Asmuth, E.J.U., Dentener, M.A., Bazil, V., Bouma, M.G., Leeuwenberg, J.F.M. and Buurman, W.A. 1993. Anti-CD14 antibodies reduce responses of cultured human endothelial cells to endotoxin. Immunol. **80**:78-83.

Vorarberger, G., Schäfer, R. and Stratowa, C. 1991. Cloning of the human gene for intercellular adhesion molecule 1 and analysis of its 5'-regulatory region. J. Immunol. **147**:2777-2786.

Vuopio-Varkila, J., Nurminen, M., Pyhälä, L. and Mäkelä, P.H. 1988. Lipopolysaccharide-induced non-specific resistance to systemic Escherichia coli infection in mice. J. Med. Microbiol. **25**:197-203.

Waage, A., Halstensen, A. and Espevik, T. 1987. Association between tumour necrosis factor in serum and fatal outcome in patients with meningococcal disease. Lancet **Feb 14, 1 (8529)**:355-357.

Walmrath, D., Ghofrani, H.A., Rosseau, S., Scutte, H., Cramer, A., Kaddus, W., Grimminger, F., Bhakdi, S. and Seeger, W. 1994. Endotoxin "priming" potentiates lung vascular abnormalities in response to Escherichia coli hemolysin: an example of synergism between endo- and exotoxin. J. Exp. Med. **180**:1437-1443.

Wang, Z., Malmberg, P., Ek, A., Larsson, K. and Palmberg, L. 1999. Swine dust induces cytokine secretion from human epithelial cells and alveolar macrophages. Clin. Exp. Immunol. **115**:6-12.

Watkins, D.N., Garlepp, M.J. and Thompson, P.J. 1997. Regulation of the inducible cyclo-oxygenase pathway in human cultured airway epithelial (A549) cells by nitric oxide. Br. J. Pharmacol. **121**:1482-1488.

Weersink, A.J.L., van Kessel, K.P.M., van den Tol, M.E., van Strijp, J.A.G., Torensma, R., Verhoef, J., Elsbach, P. and Weiss, J. 1993. Human granulocytes express a 55-kDa lipopolysaccharide-binding protein on the cell surface that is identical to the bactericidal/permeability-increasing protein. J. Immunol. **150**:253-263.

Wegner, C.D., Gundel, R.H., Reilly, P., Haynes, N., Letts, L.G. and Rothlein, R. 1990. Intercellular adhesion molecule-1 (ICAM-1) in the pathogenesis of asthma. Science **247**:456-459.

Weidemann, B., Schletter, J., Dziarski, R., Kusumoto, S., Stelter, F., Rietschel, E.T., Flad, H.-D. and Ulmer, A.J. 1997. Specific binding of soluble peptidoglycan and muramyldipeptide to CD14 on human monocytes. Infect. Immun. **65**:858-864.

Weiner, D.L., Kuppermann, N., Saladino, R.A., Thompson, C.M., Novitsky, T.J., Siber, G.R. and Fleisher, G.R. 1996. Comparison of early and late treatment with a recombinant endotoxin neutralizing protein in a rat model of Escherichia coli sepsis. Crit. Care Med. **24**:1514-1517.

Weinstein, S.L., Sanghera, J.S., Lemke, K., DeFranco, A.L. and Pelech, S.L. 1992. Bacterial lipopolysaccharide induces tyrosine phosphorylation and activation of mitogen-activated protein kinases in macrophages. J. Biol. Chem. **267**:14955-14962.

Weiss, S.J. 1989. Tissue destruction by neutrophils. N. Engl. J. Med. **320**:365-376.

Welbourn, C.R.B. and Young, Y. 1992. Endotoxin, septic shock and acute lung injury: neutrophils, macrophages and inflammatory mediators. Br. J. Surg. **79**:998-1003.

Wesselborg, S., Bauer, M.K.A., Vogt, M., Schmitz, M.L. and Schulze-Osthoff, K. 1997. Activation of transcription factor NF-κB and mitogen-activated protein kinase is mediated by distinct and separate stress effector pathways. J. Biol. Chem. **272**:12422-12429.

Westphal, O., Luderitz, O. and Bister, F. 1952. Über die Extraktion von Bakterien mit Phenol/Wasser. Z. Naturforsch. **7**:148-156.

Wheeler, A.P. and Bernard, G.R. 1999. Treating patients with severe sepsis. N. Engl. J. Med. **340**:207-214.

Whelan, J., Ghersa, P., Hooft van Huijsduijnen, R., Gray, J., Chandra, G., Talabot, F. and DeLamarter, J.F. 1991. A NF-κB-like factor is essential but not sufficient for cytokine induction of endothelial leukocyte adhesion molecule 1 (ELAM-1) gene transcription. Nucl. Acids Res. **19**:2645-2653.

Wooten, R.M., Morrison, T.B., Weis, J.H., Wright, S.D, Thieringer, R. and Weis, J.J. 1998. The role of CD14 in signaling mediated by outer membrane lipoproteins of *Borrelia burgdorferi*. J. Immunol. **160**:5485-5492.

Wright, D.G., Bralove, D.A. and Gallin, J.I. 1977. The differential mobilization of human neutrophil granules. Am. J. Pathol. **87**:273-283.

Wright, S.D., Levin, S.M., Jong, M.T.C., Chad, Z. and Kabbash, L.G. 1989. CR3 (CD11b/CD18) expresses one binding site for Arg-Gly-Asp-containing peptides and a second site for bacterial lipopolysaccharide. J. Exp. Med. **169**:175-183.

Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, R.J. and Mathison, J.C. 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science **249**:1431-1433.

Wright, S.D. 1991. Multiple receptors for endotoxin. Curr. Opin. Immunol. **3**:83–90.

Wright, S.D., Ramos, R.A., Hermanowski-Vosatka, A., Rockwell, P. and Detmers, P.A. 1991. Activation of the adhesive capacity of CR3 on neutrophils by endotoxin: dependence on lipopolysaccharide binding protein and CD14. J. Exp. Med. **173**:1281–1286.

Wright, S.D., Ramos, R.A., Patel, M. and Miller, D.S. 1992. Septin: A factor in plasma that opsonises lipopolysaccharide-bearing particles for recognition by CD14 on phagocytes. J. Exp. Med. **176**:719-722.

Wright, S.D. and Kolesnick, R.N. 1995. Does endotoxin stimulate cells by mimicking ceramide? Immunol. Today **16**:297-302.

Wurfel, M.M., Monks, B.G., Ingalls, R.R., Dedrick, R.L., Delude, R., Zhou, D., Lamping, N., Schumann, R.R., Thieringer, R., Fenton, M.J., Wright, S.D, and Golenbock, D. 1997. Targeted deletion of the lipopolysaccharide (LPS)-binding protein gene leads to profound suppression of LPS responses ex vivo, whereas in vivo responses remain intact. J. Exp. Med. **186**:2051-2056. (a)

Wurfel, M.M. and Wright, S.D. 1997. Lipopolysaccharide-binding protein and soluble CD14 transfer lipopolysaccharide to phospholipid bilayers: preferential interaction with particular classes of lipid. J. Immunol. **158**:3925-3934. (b)

Xia, P., Gamble, J.R., Rye, K.-A., Wang, L., Hii, C.S.T., Cockerill, P., Khew-Goodall, Y., Bert, A.G., Barter, P.J. and Vadas, M.A. 1998. Tumor necrosis factor-α induces adhesion molecule expression through the sphingosine kinase pathways. Proc. Natl. Acad. Sci. **95**:14196-14201.

Xing, Z., Gauldie, J., Cox, G., Baumann, H., Jordana, M., Lei, X.-F. and Achong, M.K. 1998. IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. J. Clin. Invest. **101**:311-320.

Xue, W., Kindzelskii, A.L., Todd III, R.F. and Petty, H.R. 1994. Physical association of complement receptor type 3 and urokinase-type plasminogen activator receptor in neutrophil membranes. J. Immunol. **152**:4630-4640.

Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, I., Ueno, N., Taniguchi, T., Nishida, E. and Matsumoto, K. 1995. Identification of a member of the MAPKKK family as a potential mediator of TGF-β signal transduction. Science **270**:2008-2011.

Yang, R.-B., Mark, M.R., Gray, A., Huang, A., Xie, M.H., Zhang, M., Goddard, A., Wood, W.I., Gurney, A.L. and Godowski, P.J. 1998. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. Nature **395**:284-288.

Yee, J. and Christou, N.V. 1994. The local role of tumor necrosis factor α in the modulation of neutrophil function at sites of inflammation. Arch. Surg. **129**:1249-1255.

Yu, B. and Wright, S.D. 1995. LPS-dependent interaction of Mac-2-binding protein with immobilized CD14. J. Inflamm. **45**:115-125.

Yu, B., Hailman, E. and Wright, S.D. 1997. Lipopolysaccaride-binding protein and soluble CD14 catalyze exchange of phospholipids. J. Clin. Invest. **99**:315-324.

Yuasa, T., Ohno, S., Kehrl, J.H. and Kyriakis, J.M. 1998. Tumor necrosis factor signaling to stress-activated protein kinase (SAPK/Jun NH$_2$-terminal kinase (JNK) and p38. J. Biol. Chem. **273**:22681-22692.

Zehavi-Willner, T., Barnea, A. and Pinto, M. 1991. In vivo protective effect of lipopolysaccharide against *Pseudomonas aeruginosa* exotoxin A in mice. Infect. Immun. **59**:1667-1672.

Zhang, F.X., Kirschning, C.J., Mancinelli, R., Xu, X.-P., Jin, Y., Faure, E., Mantovani, A., Rothe, M., Muzio, M. and Arditi, M. 1999. Bacterial lipopolysaccharide activates nuclear factor-κB through interleukin-1 signalling mediators in cultured human dermal endothelial cells and mononuclear phagocytes. J. Biol. Chem. **274**:7611-7614.

Ziegler, E.J., Fisher, Jr., C.J., Sprung, C.L., Straube, R.C., Sadoff, J.C., Foulke, G.E., Wortel, C.H., Fink, M.P., Dellinder, R.P., Teng, N.N.H., Allen, E., Berger, H.J., Knatterud, G.L., LoBuglio, A.F., Smith, C.R. and the HA-1A sepsis study group. 1991. Treatment of gram-negative bacteremia and septic shock with HA-1A human monoclonal antibody against endotoxin. N. Engl. J. Med. **324**:429-436.

Ziegler-Heitbrock, H.W.L and Ulevitch, R.J. 1993. CD14: cell surface receptor and differentiation marker. Immunol. Today **14**:121-125.

Dan, P., Nitzan, D.W., Dagan, A., Ginsburg, I. and Yedgar, S. 1996. H2O2 renders cells accessible to lysis by exogenous phospholipase A2: a novel mechanism for cell damage in inflammatory processes. FEBS Letters **383**:75-78.

Dishon, T., Finkel, R., Marcus, Z. and Ginsburg, I. 1967. Cell-sensitizing products of streptococci. Immunology **13**:555-564.

Ferne, M., Duchan, Z., Rabinowitz-Begner, S., Sela, M.N. and Ginsburg, I. 1978. The effect of leukocyte hydrolases on bacteria. Inflammation **3**:59-80.

Ginsburg, I., Mitra, R.S., Gibbs, D.F., Varani, J. and Kohen, R. 1993. Killing of endothelial cells and release of arachidonic acid. Inflammation **17**:295-319.

Staub, N.C., Schultz, E.L. and Albertine, K.H. 1982. Leukocytes and pulmonary microvascular injury. Ann. NY Acad. Sci. **384**:332-342.